PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference P019092WO | **FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. | |
|---|---|---|
| International application No. PCT/IB 99/ 00844 | International filing date *(day/month/year)* 27/04/1999 | (Earliest) Priority Date *(day/month/year)* 27/04/1998 |
| Applicant CHIRON S.P.A. et al. | | |

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :
      [ ] contained in the international application in written form.
      [ ] filed together with the international application in computer readable form.
      [X] furnished subsequently to this Authority in written form.
      [X] furnished subsequently to this Authority in computer readble form.
      [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
      [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has be n furnished
2. [X] **Certain claims were found unsearchable** (See Box I).
3. [ ] **Unity of invention is lacking** (see Box II).
4. With regard to the **title**,
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:
5. With regard to the **abstract**,
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rul 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the **drawings** to be published with the abstract is Figure No. 1
   [ ] as suggested by th applicant.
   [X] because the applicant failed to suggest a figure.
   [ ] because this figure better characterizes the invention.
   [ ] None of the figures.

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 99/00844

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 22 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# PATENT COOPERATION TREATY

## PCT

### NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

| | |
|---|---|
| **Date of mailing** (day/month/year)<br>02 December 1999 (02.12.99) | |
| **International application No.**<br>PCT/IB99/00844 | **Applicant's or agent's file reference**<br>P019092WO |
| **International filing date** (day/month/year)<br>27 April 1999 (27.04.99) | **Priority date** (day/month/year)<br>27 April 1998 (27.04.98) |
| **Applicant**<br>RAPPUOLI, Rino et al | |

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 November 1999 (12.11.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

| | |
|---|---|
| The International Bureau of WIPO<br>34, chemin des Colombettes<br>1211 Geneva 20, Switzerland<br>Facsimile No.: (41-22) 740.14.35 | Authorized officer<br>Marc Salzman<br>Telephone No.: (41-22) 338.83.38 |

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 99/00844

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C07K14/33 C07K14/445 C07K14/02 C07K14/11 C07K14/34 C07K14/22 C07K14/235 C12N5/10 C12N15/62 A61K39/02 A61K39/12 A61K31/715 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | THOMSON S A ET AL: "Targeting a polyepitope protein incorporating multiple class II-restricted viral epitopes to the secretory/endocytic pathway facilitates immune recognition by CD4+ cytotoxic T lymphocytes: a novel approach to vaccine design." JOURNAL OF VIROLOGY, vol. 72, no. 3, March 1998 (1998-03), pages 2246-2252, XP002128492 | 1-4,8, 17-30,32 |
| Y | figure 1<br>table 1<br>page 2248-right-hand column<br>---<br>-/-- | 10-16,31 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 January 2000 | 08/02/2000 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | ALCONADA RODRIG.., A |

5

Form PCT/ISA/210 (second sheet) (July 1992)

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP 0 429 816 A (HOFFMANN LA ROCHE) 5 June 1991 (1991-06-05) page 10, line 37-39 | 1-4,7-9, 17-22 |
| Y | tables II-IV page 11, line 40-52 | 10-16 |
| X | PARADISO, PETER R. ET AL: "Novel approaches to the development of glycoconjugate vaccines with synthetic peptides as carriers" VACCINE RES. (1993), 2(4), 239-48 , vol. 2, no. 4, 1993, pages 239-248, XP000870111 | 1-4,8, 11,12, 14-23 |
| Y | page 246 page 243 tables 2-6 figure 1 | 10-12, 14-16 |
| Y | DE VELASCO E A ET AL: "Synthetic peptides representing T-cell epitopes act as carriers in pneumococcal polysaccharide conjugate vaccines." INFECTION AND IMMUNITY, vol. 63, no. 3, March 1995 (1995-03), pages 961-968, XP002128493 cited in the application the whole document | 11, 13-16,31 |
| Y | CHRISTODOULIDES M ET AL: "Immunization with a multiple antigen peptide containing defined B- and T-cell epitopes: production of bactericidal antibodies against group B Neisseria meningitidis." MICROBIOLOGY , vol. 140, no. 11, November 1994 (1994-11), pages 2951-2960, XP000867241 page 2953-page 2954 left-hand column | 10 |
| A | WO 96 03144 A (QUEENSLAND INST MED RES ;COMMENWEALTH SCIENT AND IND RE (AU); UNIV) 8 February 1996 (1996-02-08) the whole document | 1-3, 17-21, 24-27, 30,32 |

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | AN L L ET AL: "A multivalent minigene vaccine, containing B-cell, cytotoxic T-lymphocyte and Th epitopes from several microbes, induces appropriate responses in vivo and confers protection against more than one pathogen."<br>JOURNAL OF VIROLOGY ,<br>vol. 71, no. 3, March 1997 (1997-03), pages 2292-2302, XP002128494<br>figures 1,2<br>table 1<br>see Materials and methods<br>--- | 1-9,<br>17-32 |
| A | US 4 902 506 A (ANDERSON PORTER W ET AL)<br>20 February 1990 (1990-02-20)<br>the whole document<br>--- | 11,12,<br>14-23 |
| A | US 4 882 317 A (MARBURG STEPHEN ET AL)<br>21 November 1989 (1989-11-21)<br>examples 1-10<br>--- | 11,12,<br>14-23 |
| P,X | WO 98 43677 A (CANTACUZENE DANIELE ;BAY SYLVIE (FR); LECLERC CLAUDE (FR); LO MAN)<br>8 October 1998 (1998-10-08)<br>page 11, line 21-25<br>page 13, line 20 -page 14, line 13<br>figure 1<br>examples 1-4<br>----- | 1-3,8,<br>10-14,<br>17-23 |

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 99/00844

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
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| | | AU 6557190 A | 09-05-1991 |
| | | CA 2027317 A | 01-05-1991 |
| | | JP 3173830 A | 29-07-1991 |
| | | NZ 235780 A | 28-04-1993 |
| WO 9603144 A | 08-02-1996 | AU 3072395 A | 22-02-1996 |
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| US 4882317 A | 21-11-1989 | US 4695624 A | 22-09-1987 |
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| | | KR 9102553 B | 24-04-1991 |
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| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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# ATENT COOPERATION TREAT

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference<br>P019092WO | **FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. | |
|---|---|---|
| International application No.<br>PCT/IB 99/00844 | International filing date *(day/month/year)*<br>27/04/1999 | (Earliest) Priority Date *(day/month/year)*<br>27/04/1998 |
| Applicant<br>CHIRON S.P.A. et al. | | |

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.
   [ ] filed together with the international application in computer readable form.
   [X] furnished subsequently to this Authority in written form.
   [X] furnished subsequently to this Authority in computer readble form.
   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).
3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [ ] as suggested by the applicant.
   [X] because the applicant failed to suggest a figure.
   [ ] because this figure better characterizes the invention.
   [ ] None of the figures.

Form PCT/ISA/210 (first sheet) (July 1998)

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 99/00844

**Box I Obs rvations where certain claims were found unsearchable (Continuati n of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 22 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

DUE: 28-6-00

# PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HALLYBONE, Huw George
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE

# PCT

## WRITTEN OPINION

(PCT Rule 66)

| | |
|---|---|
| Date of mailing *(day/month/year)* | 28.04.2000 |
| **REPLY DUE** | **within 2 month(s)** from the above date of mailing |

| | | |
|---|---|---|
| Applicant's or agent's file reference<br>P019092WO | | |
| International application No.<br>PCT/IB99/00844 | International filing date *(day/month/year)*<br>27/04/1999 | Priority date *(day/month/year)*<br>27/04/1998 |
| International Patent Classification (IPC) or both national classification and IPC<br>C07K14/33 | | |
| Applicant<br>CHIRON S.P.A. et al. | | |

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

   I ☒ Basis of the opinion
   II ☒ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   VI ☒ Certain document cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

   **When?** See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

   **How?** By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

   **Also:** For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis. For an informal communication with the examiner, see Rule 66.6.

   **If no reply is filed**, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 27/08/2000.

| | |
|---|---|
| Name and mailing address of the international preliminary examining authority:<br>European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Authorized officer / Examiner<br>Fotaki, M<br>Formalities officer (incl. extension of time limits)<br>Vullo, C<br>Telephone No. +49 89 2399 8061 |

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FAX +49 89 2399-4465



## Correspondence with the EPO on PCT Chapter II demands

In order to ensure that your PCT Chapter II demand is dealt with as promptly as possible you are requested to use the enclosed self-adhesive labels with any correspondence relating to the demand sent to the Munich Office.

One of these labels should be affixed to a prominent place in the upper part of the letter or form etc. which you are filing.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HALLYBONE, Huw George
CARPMAELS & RANSFORD
43 Bloomsbury Square
London WC1A 2RA
GRANDE BRETAGNE

# PCT

## NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

| | |
|---|---|
| Date of mailing (*day/month/year*) | 26.07.2000 |

| | |
|---|---|
| Applicant's or agent's file reference P019092WO | **IMPORTANT NOTIFICATION** |

| International application No. | International filing date (*day/month/year*) | Priority date (*day/month/year*) |
|---|---|---|
| PCT/IB99/00844 | 27/04/1999 | 27/04/1998 |

Applicant
CHIRON S.P.A. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

   The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

   Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

   For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

| Name and mailing address of the IPEA/ | Authorized officer |
|---|---|
| European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Christensen, J<br><br>Tel.+49 89 2399-8052 |

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| | | |
|---|---|---|
| Applicant's or agent's file reference<br>P019092WO | **FOR FURTHER ACTION** | See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) |
| International application No.<br>PCT/IB99/00844 | International filing date *(day/month/year)*<br>27/04/1999 | Priority date *(day/month/year)*<br>27/04/1998 |
| International Patent Classification (IPC) or national classification and IPC<br>C07K14/33 | | |
| Applicant<br>CHIRON S.P.A. et al. | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☒ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☒ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

| | |
|---|---|
| Date of submission of the demand<br>12/11/1999 | Date of completion of this report<br>26.07.2000 |
| Name and mailing address of the international preliminary examining authority:<br>European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Authorized officer<br>Fotaki, M<br>Telephone No. +49 89 2399 8709 |

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB99/00844

## I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**D scription, pages:**

1-58 as originally filed

**Claims, No.:**

1-32 as originally filed

**Drawings, sheets:**

1/14-14/14 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

## II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

**s e separate sheet**

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 5-7, 10-16, 31 |
| | No: | Claims | 1-4, 8, 9, 17-30, 32 |
| Inventive step (IS) | Yes: | Claims | none |
| | No: | Claims | 1-32 |
| Industrial applicability (IA) | Yes: | Claims | 1-21, 23-32 |
| | No: | Claims | 22 (reserved opinion) |

2. Citations and explanations

**see separate sheet**

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**s e separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see s parat sheet**

## II. PRIORITY

1) This international preliminary examination report has been established after consideration of the priority document GB 9808932.9 of 27.04.98 . Therefore, document WO 98 43677 published 08.10.98 cited in the International Search Report are not relevant in establishing the novelty of the present invention.

## V. REASONED STATEMENT UNDER ARTICLE 35(2)

2) The present application relates to the generation of carrier proteins comprising a number of CD4+ cell epitopes. Said proteins are meant as conjugants to capsular polysaccharides, originating from encapsulated bacteria. So conjugated polysaccharide antigens, are immunogenic and capable of eliciting a T-cell dependent immune response, thus, transforming T-cell independent polysaccharide antigens into T-cell dependent antigens useful in the preparation of conjugate vaccines suitable for protection of young children.

3) The subject-matter of **Claim 1** is not novel as required by Article 33(2) PCT.

Said claim relates to a carrier protein comprising at least five CD4+ T-cell epitopes. Documents:
D1: THOMSON S A ET AL. in JOURNAL OF VIROLOGY, vol. 72, no. 3, March 1998 (1998-03), pages 2246-2252;
D2: EP-A-0 429 816 , 5 June 1991;
disclose proteins comprising at least five CD4+ T-cell epitopes and which contain information for carrier function. Therefore the subject-matter of said claim is not novel.

Similarly, the subject-matter of **Claims 2, 3, 8, 17-30, 32** is not novel either.

4) Document D2, in particular, discloses a conjugate (Ac-Cys-$(NANP)_3)_{35}$-TT which comprises the multiple copies of the immunodominat B cell epitope of Plasmodium falciparum circumsporozoite (CS) and the full length tetanus toxoid (TT) protein. This conjugate comprises at least five different CD4+ epitopes from the TT and CS epitope. Thus, document D2 is novelty destroying for the subject-

matter of **Claims 4, 9**.

5) The subject-matter of **Claims 5-7, 10-16, 31** is not inventive as required by Article 33(3) PCT.

(i) **Claim 5** relates to a carrier protein comprising the following CD4+ epitopes: P23TT, P32TT, P21TT, PfCs, P30TT, P2TT, HBVnc, HA, HbsAg, MT.

Document D2 discloses a carrier protein comprising all the CD4+ epitopes from TT and the immunodominant epitope from CS. As mentioned in the description of the present application on page 5: "other suitable carrier peptide epitopes will be known to those of skill in the art", indicating that the selection of particular CD4+ epitopes falls within the customary practice of skilled persons. Thus, the subject-matter of **Claims 5-7, 10** lack an inventive step.

(ii) The subject-matter of **Claim 11** relates to a carrier protein as presented above, conjugated to a polysaccharide.

Documents:
D3: PARADISO, PETER R. ET AL. in VACCINE RES. (1993), 2(4), 239-48 , vol. 2, no. 4, 1993, pages 239-248;
D4: DE VELASCO E A ET AL. in INFECTION AND IMMUNITY, vol. 63, no. 3, March 1995 (1995-03), pages 961-968;
disclose carrier proteins conjugated to polysaccharides and the production of conjugate vaccines. Thus, the skilled person will combine the teachings of document D2, disclosing the carrier protein, with the teachings of any of D3 or D4, disclosing conjugates with said carrier protein and uses thereof, and he will arrive at the subject-matter of **Claims 11-16, 31** without exercising any inventive skills.

Similarly, the subject-matter of **Claims 17-30, 32** is not inventive either.

6) For the assessment of the present **Claim 22** as far as it is directed to a method of treatment of the human or animal body or to a diagnostic method practised on the human or animal body, no unified criteria exist in the PCT, on the question whether they are industrially applicable. The patentability can be dependent upon

the formulation of the claims.

## VI. CERTAIN DOCUMENTS CITED

7) The following documents are cited under Rule 70.10 PCT
WO 98 43677, published 08.10.98

## VIII. CERTAIN OBSERVATIONS ON THE INTERNATIONAL APPLICATION

8) The Applicant is reminded that the claims must be comprehensible from the technical point of view and indicate all the essential features necessary to perform the invention (Rule 6 PCT). The subject-matter of **Claims 1-3, 8, 10-32** does not fulfil this condition. Said claims are drafted as the result to be achieved, i.e. they state the technical problem rather than disclosing the technical features essential for the solution of the problem. Such a feature in the present case would be the CD4+ T cell epitopes comprised in the claimed protein.

9) Furthermore, dependent **Claims 4-7, 9** do not clearly specify the claimed subject-matter contrary to the requirements of Article 6 PCT. The claimed epitope or protein is only defined by an arbitrary designation, namely "P23TT, P32TT, P21TT, PfCs, P30TT, P2TT, HBVnc, HA, HbsAg, MT, hsp70, N6, N10 or N19" without disclosing any technical feature which unambiguously characterizes the claimed subject-matter. An epitope or a protein being a chemical product should be clearly defined by its formula i.e. its amino acid sequence as for example shown on Table I page 36 and Figures 1, 2 and 8.

Special note is made to the fact that an epitope with the name "PfCs" is not disclosed by the description, thus, the subject-matter of **Claims 4-7** does not meet the requirements of Article 5 PCT.

10) The vague and imprecise statement "incorporated herein by reference" in the description, on page 31 for example, implies that the subject-matter for which protection is sought may be different than that defined by the claims, thereby, resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, PCT/GL/3 III, 4.3a).

## I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".)*:

**Description, pages:**

1-58 as originally filed

**Claims, No.:**

1-32 as originally filed

**Drawings, sheets:**

1/14-14/14 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

## II. Priority

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

   **see separate sheet**

**V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | |
|---|---|---|
| Novelty (N) | Claims | 1-4, 8, 9, 17-30, 32 |
| Inventive step (IS) | Claims | 1-32 |
| Industrial applicability (IA) | Claims | 22 (reserved opinion) |

2. Citations and explanations

   **see separate sheet**

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

   and / or

2. Non-written disclosures (Rule 70.9)

   **see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

## II. PRIORITY

1) This first preliminary written opinion has been established after consideration of the priority document GB 9808932.9 of 27.04.98 . Therefore, document WO 98 43677 published 08.10.98 cited in the International Search Report are not relevant in establishing the novelty of the present invention.

## V. REASONED STATEMENT UNDER RULE 66.2 (a) (ii)

2) The present application relates to the generation of carrier proteins comprising a number of CD4+ cell epitopes. Said proteins are meant as conjugants to capsular polysaccharides, originating from encapsulated bacteria. So conjugated polysaccharide antigens, are immunogenic and capable of eliciting a T-cell dependent immune response, thus, transforming T-cell independent polysaccharide antigens into T-cell dependent antigens useful in the preparation of conjugate vaccines suitable for protection of young children.

3) The subject-matter of **Claim 1** is not novel as required by Article 33(2) PCT.

Said claim relates to a carrier protein comprising at least five CD4+ T-cell epitopes. Documents:
D1: THOMSON S A ET AL. in JOURNAL OF VIROLOGY, vol. 72, no. 3, March 1998 (1998-03), pages 2246-2252;
D2: EP-A-0 429 816 , 5 June 1991;
disclose proteins comprising at least five CD4+ T-cell epitopes and which contain information for carrier function. Therefore the subject-matter of said claim is not novel.

Similarly, the subject-matter of **Claims 2, 3, 8, 17-30, 32** is not novel either.

4) Document D2, in particular, discloses a conjugate (Ac-Cys-$(NANP)_3)_{35}$-TT which comprises the multiple copies of the immunodominat B cell epitope of Plasmodium falciparum circumsporozoite (CS) and the full length tetanus toxoid (TT) protein. This conjugate comprises at least five different CD4+ epitopes from the TT and CS epitope. Thus, document D2 is novelty destroying for the subject-

matter of **Claims 4, 9.**

5) The subject-matter of **Claims 5-7, 10-16, 31** is not inventive as required by Article 33(3) PCT.

(i) **Claim 5** relates to a carrier protein comprising the following CD4+ epitopes: P23TT, P32TT, P21TT, PfCs, P30TT, P2TT, HBVnc, HA, HbsAg, MT.

Document D2 discloses a carrier protein comprising all the CD4+ epitopes from TT and the immunodominant epitope from CS. As mentioned in the description of the present application on page 5: "other suitable carrier peptide epitopes will be known to those of skill in the art", indicating that the selection of particular CD4+ epitopes falls within the customary practice of skilled persons. Thus, the subject-matter of **Claims 5-7, 10** lack an inventive step.

(ii) The subject-matter of **Claim 11** relates to a carrier protein as presented above, conjugated to a polysaccharide.

Documents:

D3: PARADISO, PETER R. ET AL. in VACCINE RES. (1993), 2(4), 239-48 , vol. 2, no. 4, 1993, pages 239-248;

D4: DE VELASCO E A ET AL. in INFECTION AND IMMUNITY, vol. 63, no. 3, March 1995 (1995-03), pages 961-968;

disclose carrier proteins conjugated to polysaccharides and the production of conjugate vaccines. Thus, the skilled person will combine the teachings of document D2, disclosing the carrier protein, with the teachings of any of D3 or D4, disclosing conjugates with said carrier protein and uses thereof, and he will arrive at the subject-matter of **Claims 11-16, 31** without exercising any inventive skills.

Similarly, the subject-matter of **Claims 17-30, 32** is not inventive either.

6) For the assessment of the present **Claim 22** as far as it is directed to a method of treatment of the human or animal body or to a diagnostic method practised on the human or animal body, no unified criteria exist in the PCT, on the question whether they are industrially applicable. The patentability can be dependent upon

the formulation of the claims.

## VI. CERTAIN DOCUMENTS CITED

7) The following documents are cited under Rule 70.10 PCT
WO 98 43677, published 08.10.98

## VIII. CERTAIN OBSERVATIONS ON THE INTERNATIONAL APPLICATION

8) The Applicant is reminded that the claims must be comprehensible from the technical point of view and indicate all the essential features necessary to perform the invention (Rule 6 PCT). The subject-matter of **Claims 1-3, 8, 10-32** does not fulfil this condition. Said claims are drafted as the result to be achieved, i.e. they state the technical problem rather than disclosing the technical features essential for the solution of the problem. Such a feature in the present case would be the CD4+ T cell epitopes comprised in the claimed protein.

9) Furthermore, dependent **Claims 4-7, 9** do not clearly specify the claimed subject-matter contrary to the requirements of Article 6 PCT. The claimed epitope or protein is only defined by an arbitrary designation, namely "P23TT, P32TT, P21TT, PfCs, P30TT, P2TT, HBVnc, HA, HbsAg, MT, hsp70, N6, N10 or N19" without disclosing any technical feature which unambiguously characterizes the claimed subject-matter. An epitope or a protein being a chemical product should be clearly defined by its formula i.e. its amino acid sequence as for example shown on Table I page 36 and Figures 1, 2 and 8.

Special note is made to the fact that an epitope with the name "PfCs" is not disclosed by the description, thus, the subject-matter of **Claims 4-7** does not meet the requirements of Article 5 PCT.

10) The vague and imprecise statement "incorporated herein by reference" in the description, on page 31 for example, implies that the subject-matter for which protection is sought may be different than that defined by the claims, thereby, resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, PCT/GL/3 III, 4.3a).

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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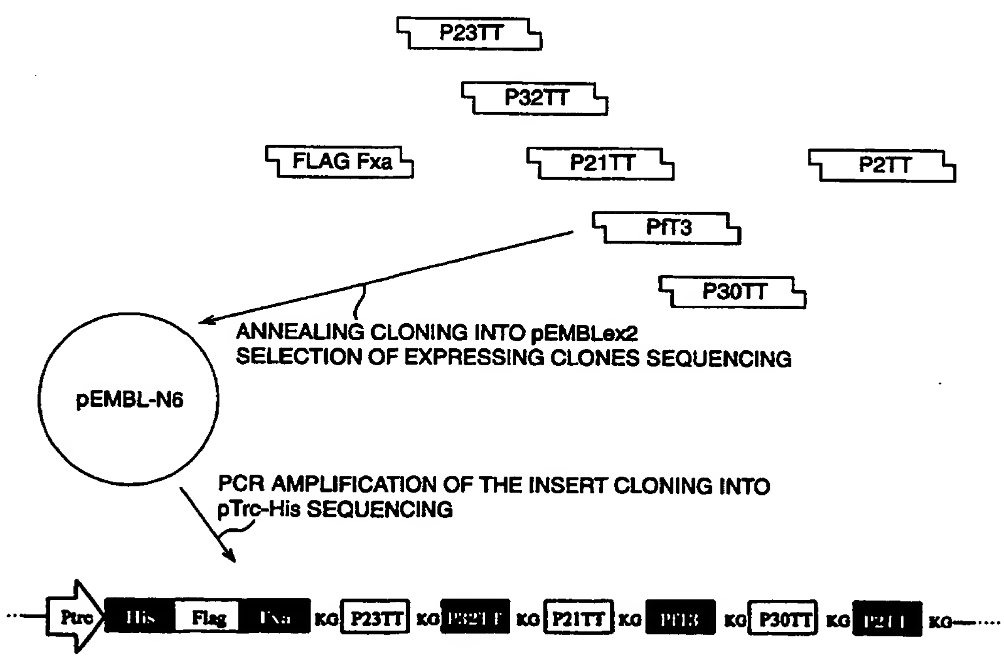
(54) Title: POLYEPITOPE CARRIER PROTEIN




(57) Abstract

The invention relates to polyepitope carrier proteins that comprise at least five CD4+ T cell epitopes, for conjugation to capsular polysaccharides. The carrier proteins are use useful as components of vaccines that can elicit a T–cell dependent immune response. These vaccines are particularly useful to confer protection against infection from encapsulated bacteria in infants between the ages of 3 months and about 2 years.

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# Polyepitope Carrier Protein

The present invention relates to polyepitope carrier proteins. When conjugated to capsular polysaccharides, these carrier proteins are useful as components of vaccines that are capable of eliciting a T-cell dependent immune response. Particularly, the proteins of the present invention may be used to confer protection against infection from encapsulated bacteria in infants between the ages of 3 months and about 2 years.

Encapsulated bacteria such as *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* constitute a significant cause of morbidity and mortality in neonates and infants world-wide (Tunkel & Scheld, 1993). In developing countries, around one million children die each year due to pneumonia alone. Furthermore, even in developed countries, the increase in the phenomenon of antibiotic resistance means that there is a great need to improve on existing vaccines.

The polysaccharide capsule of *H. influenzae, N. meningitidis and S. pneumoniae* represents a major virulence factor that is important for nasopharyngeal colonisation and systemic invasion by encapsulated bacteria (Moxon and Kroll, 1990). Consequently, much of the research directed towards finding protective immunogens has focused on capsular polysaccharides. The finding that these polysaccharides are able to elicit the formation of protective antibodies led to the development of a number of vaccines that have been efficacious in protecting adults from disease (Andreoni *et al.* 1993; Goldblatt *et al.* 1992).

The problem with capsular polysaccharide vaccines developed to date is that they suffer an inherent inability to protect children under two years of age from disease (Holmes and Granoff 1992). This is a significant drawback when it is appreciated that this population of children is at highest risk of infection. Their failure to block infection is believed to derive from the T-cell independent (TI) type of immune reaction that is the only antibody response used by the body against polysaccharide antigens. This type of response does not involve MHC Class II restriction molecules for antigen presentation to T-cells; as a consequence, T-cell help is prevented. Although the TI response works

well in adults, it is inactive in very young children due to a combination of factors such as functional B-cell immaturity, inactivation of B-cell receptor-mediated signalling and B-cell anergy in response to antigen stimulation.

To overcome this drawback, two particular vaccine approaches are currently being investigated. The first is the development of anti-idiotype vaccines that contain peptides that mimic carbohydrate idiotypes (McNamara 1984; Agadjanyan, 1997). The second approach involves conjugate vaccines that are designed to transform T-cell independent (TI) polysaccharide antigens into T-dependent (TD) antigens through the covalent linkage of the polysaccharide to a peptide carrier.

*H. influenzae* type B (Hib) conjugate vaccines represent a leading example for the development of other vaccines against infections that are due to capsulated bacteria. In fact, meningitis and other infections caused by Hib have declined dramatically in countries where widespread vaccination with Hib conjugate has been achieved (Robins, 1996). Complete elimination of the pathogen might be possible, but depends upon several factors, including a further improvement of the existing vaccines (Liptak, 1997).

The widely distributed paediatric vaccine antigens tetanus and diptheria toxoids have been selected as carrier proteins with the aim of taking advantage of an already-primed population at the time of conjugate vaccine injection. Previous vaccination with paediatric diptheria-tetanus (DT) or diptheria-tetanus-pertussis (DTP) vaccines means that carrier priming may now be exploited to enhance the immune response to polysaccharide conjugates.

A number of such vaccines have been successfully produced and have been efficacious in reducing the number of deaths caused by these pathogens. The carriers used in these vaccines are large antigens such as tetanus toxoid, non-toxic diptheria toxin mutant CRM197 and group B *N. meningitidis* outer membrane protein complex (OMPC). However, in the future, it is thought that as the number of conjugate vaccines containing the same carrier proteins increases, the suppression of immune responses by pre-existing antibodies to the carrier is likely to become a problem.

Much research is now being directed to the development of improved carrier molecules that contain carrier peptides comprising CD4+ T helper cell (Th) epitopes, but which do

not possess T-cell suppressive (Ts) functions (Etlinger *et al.* 1990). Peptides which retain only helper functions (CD4+ epitopes) are most suitable as carriers, since their effect is sufficient to induce T cell help but the carrier is small enough to limit or to completely avoid production of anti-carrier antibodies.

Various publications demonstrate the ability of such peptides to confer T-cell help to haptens when covalently linked to them (Etlinger, 1990; Valmori 1992; Sadd 1992; Kumar 1992; Kaliyaperumal, 1995; De Velasco, 1995 and Bixler 1989). However, to date, these publications have not resulted in the development of effective vaccines. There thus remains a great need for the development of new, improved vaccine strategies that are effective in combating diseases caused by encapsulated bacteria in infants and young children.

## SUMMARY OF THE INVENTION

According to the present invention, there is provided a carrier protein comprising at least five CD4+ T-cell epitopes. Preferably, the carrier protein is conjugated to a polysaccharide. These compounds are useful as immunogenic compounds that may in turn be useful as components of protective vaccines against diseases caused by bacterial pathogens.

A carrier protein is an antigenic polypeptide entity that induces the formation of antibodies directed against an antigen conjugated to it, by the immune system of an organism into which the carrier-antigen conjugate is introduced. The necessity to use carrier proteins results from the fact that although many short epitopes are protective, they are poorly immunogenic. This negates the usefulness of these epitopes in the generation of new and efficacious vaccines. By conjugating an immunogenic carrier protein to a molecule that is non-immunogenic, it is possible to confer the high immunogenicity of the carrier protein onto the conjugate molecule. Such conjugate molecules stimulate the generation of an immune response against the non-immunogenic portion of the conjugate molecule and thus have been effectively used in vaccines that protect against pathogens for which protective immunity could not otherwise be generated.

Hence, highly immunogenic proteins (such as tetanus toxoid) have historically been used as carriers in order to induce a Th cell response that provides help to B cells for the production of antibodies directed against non-immunogenic epitopes. However, overall effectiveness has not been generally achieved with this approach, since the antibody response to a hapten (the epitope) coupled to a carrier protein can be inhibited when the recipient host has been previously immunised with the unmodified carrier protein. This phenomenon is termed epitope-specific suppression and has now been studied in a variety of hapten-carrier systems.

Coupling of bacterial polysaccharides to carrier proteins has been shown to improve the immunogenicity of the polysaccharide and results in antigens with novel characteristics. Furthermore, the coupling of a thymus-independent (TI) polysaccharide to a protein makes the polysaccharide thymus-dependent (TD).

A CD4+ T cell epitope is a peptide epitope that stimulates the activity of those T cells that are MHC Class II restricted. This subset of T cells includes Th cells. Many CD4+ epitopes are well known to those of skill in the art and have been shown to confer T cell help to haptens when covalently attached to them (Etlinger *et al*, 1990; Valmori 1992; Sadd 1992; Kumar 1992; Kaliyaperumal, 1995).

The CD4+ T epitopes used in the carrier proteins of the present invention ideally comprise peptides that are of as short a length as possible. The epitope will thus retain its characteristics to a sufficient degree to induce T-cell help, yet will be small enough that excessive production of anti-carrier antibodies will be minimised. This is preferable, since it is thought that suppression of immune responses by pre-existing antibodies to carrier epitopes is likely to become a problem in the future if the number of congregate vaccines containing common carrier proteins keeps growing. Furthermore, the use of short peptides as carrier epitopes affords the rational selection of suitable Th epitopes, whilst avoiding stretches of sequence that contain B-cell or T-suppressor epitopes that will be detrimental to the function of the protein in eliciting a TI immune response.

Suitable proteins from which CD4+ epitopes may be selected include tetanus toxin (TT), *Plasmodium falciparum* circumsporozite, hepatitis B surface antigen, hepatitis B nuclear core protein, *H. influenzae* matrix protein, *H. influenzae* haemagglutinin,

diphtheria toxoid, diphtheria toxoid mutant CRM197, group B *N. meningitidis* outer membrane protein complex (OMPC), the pneumococcal toxin pneumolysin, and heat shock proteins from *Mycobacterium bovis* and *M. leprae*. The *M. leprae* HSP70 408-427 epitope is not found in the corresponding human homologous sequence (Adams et al., 1997 *Infect Immun*, 65: 1061-70); since a possible limitation in the use of HSP motifs in vaccine formulations is the possibility to induce autoimmune responses due to the high homology between microbial and human HSPs, this epitope is particularly preferred. Other suitable carrier peptide epitopes will be well known to those of skill in the art. The CD4+ T-cell epitopes selected from these antigens are recognised by human CD4+ T cells.

It has been found that the number of T-cell epitopes present in the carrier protein has a significant influence in conferring T-cell help to oligosaccharide molecules conjugated thereto. The polyepitope carrier protein should contain five or more CD4+ T-cell epitopes. Preferably, the polyepitope carrier protein contains between 5 and 50 degenerate CD4+ T-cell epitopes, more preferably between 5 and 20 epitopes, even more preferably 5, 6, 7, 8, 9, 10, 11 or 19 degenerate CD4+ T-cell epitopes. The use of a number of universal epitopes in the carrier protein has been found to reduce the problem of genetic restriction of the immune response generated against peptide antigens.

In addition to CD4+ epitopes, the carrier proteins of the present invention may comprise other peptides or protein fragments, such as epitopes from immunomodulating cytokines such as interleukin-2 (IL-2) or granulocyte-macrophage colony stimulating factor (GM-CSF). Promiscuous peptides (Panina-Bordignon *et al* 1989), the so-called "universal" peptides (Kumar *et al.*, 1992), cluster peptides (Ahlers *et al.*, 1993) or peptides containing both T cell and B cell epitopes (Lett *et al*, 1994) may also be used to recruit various effector systems of the immune system, as required.

The polyepitope carrier protein may be produced by any suitable means, as will be apparent to those of skill in the art. Two preferred methods of construction of carrier proteins according to the invention are direct synthesis and by production of recombinant protein. Preferably, the polyepitope carrier proteins of the present invention are produced by recombinant means, by expression from an encoding nucleic acid molecule. Recombinant expression has the advantage that the production of the

carrier protein is inexpensive, safe, facile and does not involve the use of toxic compounds that may require subsequent removal.

When expressed in recombinant form, the carrier proteins of the present invention are generated by expression from an encoding nucleic acid in a host cell. Any host cell may be used, depending upon the individual requirements of a particular vaccine system. Preferably, bacterial hosts are used for the production of recombinant protein, due to the ease with which bacteria may be manipulated and grown. The bacterial host of choice is *Escherichia coli*.

Preferably, if produced recombinantly, the carrier proteins are expressed from plasmids that contain a synthetic nucleic acid insert. Such inserts may be designed by annealing oligonucleotide duplexes that code for the CD4+ T-cell epitopes. The 5'and 3' ends of the synthetic linkers may be designed so as to anneal to each other. This technique allows annealing of the oligonucleotides in a random order, resulting in a mixture of potentially different mini-genes comprising any one of a number of possible combinations of epitopes. This mixture is then cloned into any suitable expression vector and a selection process of expressing clones is then performed. This strategy ensures that only those clones are selected that produce a carrier protein that is not detrimental to the health of the cell in which it is expressed. Conversely, arbitrary selection of the order of epitopes has been found to be less successful.

The ends of the epitope-encoding linkers may be designed so that two codons are introduced between the individual epitopes when annealing takes place. Amino acid residues such as glycine or lysine are examples of suitable residues for use in the spacers. In particular, the use of lysine residues in spacers allows the further congregation of carrier protein to capsular polysaccharide. Additionally, the insertion site in the expression plasmid into which the nucleic acid encoding carrier protein is cloned may allow linkage of the polyepitope carrier protein to a tag, such as the "flag" peptide or polyhistidine. This arrangement facilitates the subsequent purification of recombinant protein.

Nucleic acid encoding the polyepitope carrier protein may be cloned under the control of an inducible promoter, so allowing precise regulation of carrier protein expression.

Suitable inducible systems will be well known to those of skill in the art and include the well-known *lac* system (Sambrook *et al.* 1989).

Methods of recombinant expression of carrier proteins according to the invention will be well known to the skilled artisan, but for the purposes of clarity are briefly discussed herein.

Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation [Sambrook *et al.* (1989) "Expression of Cloned Genes in Mammalian Cells." In *Molecular Cloning: A Laboratory Manual, 2nd ed.*].

Mammalian viral genes are often highly expressed and have a broad host range; therefore sequences encoding mammalian viral genes provide particularly useful promoter sequences. Examples include the SV40 early promoter, mouse mammary tumour virus LTR promoter, adenovirus major late promoter (Ad MLP), and herpes simplex virus promoter. In addition, sequences derived from non-viral genes, such as the murine metallotheionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated (inducible), depending on the promoter can be induced with glucocorticoid in hormone-responsive cells.

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a regulatory DNA sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from

the transcription initiation site, in either normal or flipped orientation, or at a distance of more than 1000 nucleotides from the promoter [Maniatis *et al.* (1987) *Science 236*:1237; Alberts *et al.* (1989) *Molecular Biology of the Cell*, 2nd ed.]. Enhancer elements derived from viruses may be particularly useful, because they usually have a broader host range. Examples include the SV40 early gene enhancer [Dijkema *et al* (1985) *EMBO J. 4*:761] and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus [Gorman *et al.* (1982b) *Proc. Natl. Acad. Sci. 79*:6777] and from human cytomegalovirus [Boshart *et al.* (1985) *Cell 41*:521]. Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or metal ion [Sassone-Corsi and Borelli (1986) *Trends Genet. 2*:215; Maniatis *et al.* (1987) Science 236:1237].

A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus triparite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Usually, transcription termination and polyadenylation sequences recognised by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation [Birnstiel *et al.* (1985) *Cell 41*:349; Proudfoot and Whitelaw (1988) "Termination and 3' end processing of eukaryotic RNA. In *Transcription and splicing* (ed. B.D. Hames and D.M. Glover); Proudfoot (1989) *Trends Biochem. Sci. 14*:105].

These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminater/polyadenylation signals include those derived from SV40 [Sambrook *et al* (1989) "Expression of cloned genes in cultured mammalian cells." In *Molecular Cloning: A Laboratory Manual*].

Some genes may be expressed more efficiently when introns (also called intervening sequences) are present. Several cDNAs, however, have been efficiently expressed from vectors that lack splicing signals (also called splice donor and acceptor sites) [see e.g., Gothing and Sambrook (1981) *Nature 293*:620]. Introns are intervening noncoding sequences within a coding sequence that contain splice donor and acceptor sites. They are removed by a process called "splicing," following polyadenylation of the primary transcript [Nevins (1983) *Annu. Rev. Biochem. 52*:441; Green (1986) *Annu. Rev. Genet. 20*:671; Padgett *et al.* (1986) *Annu. Rev. Biochem. 55*:1119; Krainer and Maniatis (1988) "RNA splicing." In *Transcription and splicing* (ed. B.D. Hames and D.M. Glover)].

Usually, the above-described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those derived from animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 [Gluzman (1981) *Cell 23*:175] or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen. Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replicaton systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a prokaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 [Kaufman *et al.* (1989) *Mol. Cell. Biol. 9*:946 and pHEBO [Shimizu *et al.* (1986) *Mol. Cell. Biol. 6*:1074].

The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalised cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), and a number of other cell lines.

The polynucleotide encoding the protein can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction employs techniques that are known in the art. Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene or genes to be expressed; a wild type baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene in to the baculovirus genome); and appropriate insect host cells and growth media.

After inserting the DNA sequence encoding the protein into the transfer vector, the vector and the wild type viral genome are transfected into an insect host cell where the vector and viral genome are allowed to recombine. The packaged recombinant virus is expressed and recombinant plaques are identified and purified. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987) (hereinafter "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein into the baculovirus genome, the above described components, comprising a promoter, leader (if desired), coding

sequence of interest, and transcription termination sequence, are usually assembled into an intermediate transplacement construct (transfer vector). This construct may contain a single gene and operably linked regulatory elements; multiple genes, each with its owned set of operably linked regulatory elements; or multiple genes, regulated by the same set of regulatory elements. Intermediate transplacement constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as a bacterium. The replicon will have a replication system, thus allowing it to be maintained in a suitable host for cloning and amplification.

Currently, the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT; see Luckow and Summers, *Virology* (1989) *17*:31.

The plasmid usually also contains the polyhedrin polyadenylation signal (Miller *et al.* (1988) *Ann. Rev. Microbiol.*, *42*:177) and a prokaryotic ampicillin-resistance (*amp*) gene and origin of replication for selection and propagation in *E. coli*.

Baculovirus transfer vectors usually contain a baculovirus promoter. A baculovirus promoter is any DNA sequence capable of binding a baculovirus RNA polymerase and initiating the downstream (5' to 3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A baculovirus transfer vector may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

Structural genes, abundantly transcribed at late times in a viral infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron protein, Friesen *et al.*, (1986) "The Regulation of Baculovirus Gene Expression," in: *The Molecular Biology of Baculoviruses* (ed. Walter

Doerfler); EPO Publ. Nos. 127 839 and 155 476; and the gene encoding the p10 protein, Vlak *et al.*, (1988), *J. Gen. Virol. 69*:765.

DNA encoding suitable signal sequences can be derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell *et al.* (1988) *Gene*, *73*:409). Alternatively, since the signals for mammalian cell posttranslational modifications (such as signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognised by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human γ-interferon, Maeda *et al.*, (1985), *Nature 315*:592; human gastrin-releasing peptide, Lebacq-Verheyden *et al.*, (1988), *Molec. Cell. Biol. 8*:3129; human IL-2, Smith *et al.*, (1985) *Proc. Nat'l Acad. Sci. USA*, *82*:8404; mouse IL-3, (Miyajima *et al.*, (1987) *Gene 58*:273; and human glucocerebrosidase, Martin *et al.* (1988) *DNA*, *7*:99, can also be used to provide for secretion in insects.

A recombinant polypeptide or polyprotein may be expressed intracellularly or, if it is expressed with the proper regulatory sequences, it can be secreted. Good intracellular expression of non-fused foreign proteins usually requires heterologous genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. If desired, methionine at the N-terminus may be cleaved from the mature protein by *in vitro* incubation with cyanogen bromide.

Alternatively, recombinant polyproteins or proteins which are not naturally secreted can be secreted from the insect cell by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in insects. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the translocation of the protein into the endoplasmic reticulum.

After insertion of the DNA sequence and/or the gene encoding the expression product precursor of the protein, an insect cell host is co-transformed with the heterologous DNA of the transfer vector and the genomic DNA of wild type baculovirus - usually by co-transfection. The promoter and transcription termination sequence of the construct

will usually comprise a 2-5kb section of the baculovirus genome. Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summers and Smith *supra*; Ju *et al.* (1987); Smith *et al.*, *Mol. Cell. Biol.* (1983) *3*:2156; and Luckow and Summers (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene. Miller *et al.*, (1989), *Bioessays 4*:91.The DNA sequence, when cloned in place of the polyhedrin gene in the expression vector, is flanked both 5' and 3' by polyhedrin-specific sequences and is positioned downstream of the polyhedrin promoter.

The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus. Homologous recombination occurs at low frequency (between about 1% and about 5%); thus, the majority of the virus produced after cotransfection is still wild-type virus. Therefore, a method is necessary to identify recombinant viruses. An advantage of the expression system is a visual screen allowing recombinant viruses to be distinguished. The polyhedrin protein, which is produced by the native virus, is produced at very high levels in the nuclei of infected cells at late times after viral infection. Accumulated polyhedrin protein forms occlusion bodies that also contain embedded particles. These occlusion bodies, up to 15 □m in size, are highly refractile, giving them a bright shiny appearance that is readily visualised under the light microscope. Cells infected with recombinant viruses lack occlusion bodies. To distinguish recombinant virus from wild-type virus, the transfection supernatant is plaqued onto a monolayer of insect cells by techniques known to those skilled in the art. Namely, the plaques are screened under the light microscope for the presence (indicative of wild-type virus) or absence (indicative of recombinant virus) of occlusion bodies. "Current Protocols in Microbiology" Vol. 2 (Ausubel *et al.* eds) at 16.8 (Supp. 10, 1990); Summers and Smith, *supra*; Miller *et al.* (1989).

Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, *inter alia*: *Aedes aegypti* , *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni* (PCT Pub. No. WO 89/046699; Carbonell *et al.*, (1985) *J. Virol. 56*:153; Wright (1986) *Nature 321*:718;

Smith *et al.*, (1983) *Mol. Cell. Biol. 3*:2156; and see generally, Fraser, *et al.* (1989) *In Vitro Cell. Dev. Biol.. 25*:225).

Cells and cell culture media are commercially available for both direct and fusion expression of heterologous polypeptides in a baculovirus/expression system; cell culture technology is generally known to those skilled in the art. *See, e.g.*, Summers and Smith *supra*.

The modified insect cells may then be grown in an appropriate nutrient medium, which allows for stable maintenance of the plasmid(s) present in the modified insect host. Where the expression product gene is under inducible control, the host may be grown to high density, and expression induced. Alternatively, where expression is constitutive, the product will be continuously expressed into the medium and the nutrient medium must be continuously circulated, while removing the product of interest and augmenting depleted nutrients. The product may be purified by such techniques as chromatography, e.g., HPLC, affinity chromatography, ion exchange chromatography, etc.; electrophoresis; density gradient centrifugation; solvent extraction, or the like. As appropriate, the product may be further purified, as required, so as to remove substantially any insect proteins which are also secreted in the medium or result from lysis of insect cells, so as to provide a product which is at least substantially free of host debris, e.g., proteins, lipids and polysaccharides.

In order to obtain protein expression, recombinant host cells derived from the transformants are incubated under conditions which allow expression of the recombinant protein encoding sequence. These conditions will vary, dependent upon the host cell selected. However, the conditions are readily ascertainable to those of ordinary skill in the art, based upon what is known in the art.

Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3") transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also

have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the lac operon in Escherichia coli (E. coli) [Raibaud *et al.* (1984) *Annu. Rev. Genet. 18*:173]. Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.

Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolising enzymes, such as galactose, lactose (*lac*) [Chang *et al.* (1977) *Nature 198*:1056], and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*) [Goeddel *et al.* (1980) *Nuc. Acids Res. 8*:4057; Yelverton *et al.* (1981) *Nucl. Acids Res. 9*:731; U.S. Patent No. 4,738,921; EPO Publ. Nos. 036 776 and 121 775]. The g-laotamase (*bla*) promoter system [Weissmann (1981) "The cloning of interferon and other mistakes." In *Interferon 3* (ed. I. Gresser)], bacteriophage lambda PL [Shimatake *et al.* (1981) *Nature 292*:128] and T5 [U.S. Patent No. 4,689,406] promoter systems also provide useful promoter sequences.

In addition, synthetic promoters that do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter [U.S. Patent No. 4,551,433]. For example, the *tac* promoter is a hybrid *trp-lac* promoter comprised of both *trp* promoter and *lac* operon sequences that is regulated by the *lac* repressor [Amann *et al.* (1983) *Gene 25*:167; de Boer *et al.* (1983) *Proc. Natl. Acad. Sci. 80*:21]. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of

some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an example of a coupled promoter system [Studier *et al.* (1986) *J. Mol. Biol. 189*:113; Tabor *et al.* (1985) *Proc Natl. Acad. Sci. 82*:1074]. In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an *E. coli* operator region (EPO Publ. No. 267 851).

In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In *E. coli*, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon [Shine *et al.* (1975) *Nature 254*:34]. The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' and of *E. coli* 16S rRNA [Steitz *et al.* (1979) "Genetic signals and nucleotide sequences in messenger RNA." In *Biological Regulation and Development: Gene Expression* (ed. R.F. Goldberger)]. To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site [Sambrook *et al.* (1989) "Expression of cloned genes in Escherichia coli." In *Molecular Cloning: A Laboratory Manual*].

A DNA molecule may be expressed intracellularly. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide or by either *in vivo* on *in vitro* incubation with a bacterial methionine N-terminal peptidase (EPO Publ. No. 219 237).

Fusion proteins provide an alternative to direct expression. Usually, a DNA sequence encoding the N-terminal portion of an endogenous bacterial protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the bacteriophage lambda cell gene can be linked at the 5' terminus of a foreign gene and expressed in bacteria. The resulting fusion protein preferably retains a site for a processing enzyme (factor Xa) to cleave the bacteriophage protein from the foreign gene [Nagai *et al.* (1984) *Nature 309*:810]. Fusion proteins can also be made with

sequences from the *lacZ* [Jia *et al.* (1987) *Gene 60*:197], *trpE* [Allen *et al.* (1987) J. Biotechnol. 5:93; Makoff *et al.* (1989) *J. Gen. Microbiol. 135*:11], and *Chey* [EPO Publ. No. 324 647] genes. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (e.g. ubiquitin specific processing-protease) to cleave the ubiquitin from the foreign protein. Through this method, native foreign protein can be isolated [Miller *et al.* (1989) *Bio/Technology 7*:698].

Alternatively, foreign proteins can also be secreted from the cell by creating chimeric DNA molecules that encode a fusion protein comprised of a signal peptide sequence fragment that provides for secretion of the foreign protein in bacteria [U.S. Patent No. 4,336,336]. The signal sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). Preferably there are processing sites, which can be cleaved either *in vivo* or *in vitro* encoded between the signal peptide fragment and the foreign gene.

DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the *E. coli* outer membrane protein gene (*ompA*) [Masui *et al.* (1983), in: *Experimental Manipulation of Gene Expression*; Ghrayeb *et al.* (1984) *EMBO J. 3*:2437] and the *E. coli* alkaline phosphatase signal sequence (*phoA*) [Oka *et al.* (1985) *Proc. Natl. Acad. Sci. 82*:7212]. As an additional example, the signal sequence of the alpha-amylase gene from various Bacillus strains can be used to secrete heterologous proteins from *B. subtilis* [Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:5582; EPO Publ. No. 244 042].

Usually, transcription termination sequences recognised by bacteria are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Transcription termination sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include

transcription termination sequences derived from genes with strong promoters, such as the *trp* gene in *E. coli* as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will have a replication system, thus allowing it to be maintained in a prokaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various Bacillus strains integrate into the Bacillus chromosome (EPO Publ. No. 127 328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline [Davies *et al.* (1978) *Annu. Rev. Microbiol. 32*:469]. Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways.

Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable market that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extra-chromosomal replicons or integrating vectors, have been developed for transformation into many bacteria. For example, expression vectors have been developed for, *inter alia*, the following bacteria: Bacillus subtilis [Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541], Escherichia coli [Shimatake *et al.* (1981) *Nature 292*:128; Amann *et al.* (1985) *Gene 40*:183; Studier *et al.* (1986) *J. Mol. Biol. 189*:113; EPO Publ. Nos. 036 776, 136 829 and 136 907], Streptococcus cremoris [Powell *et al.* (1988) *Appl. Environ. Microbiol. 54*:655]; Streptococcus lividans [Powell *et al.* (1988) *Appl. Environ. Microbiol. 54*:655], Streptomyces lividans [U.S. Patent No. 4,745,056].

Methods of introducing exogenous DNA into bacterial hosts are well-known in the art, and usually include either the transformation of bacteria treated with $CaCl_2$ or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation. Transformation procedures usually vary with the bacterial species to be transformed. See e.g., [Masson *et al.* (1989) *FEMS Microbiol. Lett. 60*:273; Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541, Bacillus], [Miller *et al.* (1988) *Proc. Natl. Acad. Sci. 85*:856; Wang *et al.* (1990) *J. Bacteriol. 172*:949, Campylobacter], [Cohen *et al.* (1973) *Proc. Natl. Acad. Sci. 69*:2110; Dower *et al.* (1988) *Nucleic Acids Res. 16*:6127; Kushner (1978) "An improved method for transformation of Escherichia coli with ColE1-derived plasmids. In *Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering* (eds. H.W. Boyer and S. Nicosia); Mandel *et al.* (1970) *J. Mol. Biol. 53*:159; Taketo (1988) *Biochim. Biophys. Acta 949*:318; Escherichia], [Chassy *et al.* (1987) *FEMS Microbiol. Lett. 44*:173 Lactobacillus]; [Fiedler *et al.* (1988) *Anal. Biochem 170*:38, Pseudomonas]; [Augustin *et al.* (1990) *FEMS Microbiol. Lett. 66*:203, Staphylococcus], [Barany *et al.* (1980) *J. Bacteriol. 144*:698; Harlander (1987) "Transformation of Streptococcus lactis by electroporation, in: *Streptococcal Genetics* (ed. J. Ferretti and R. Curtiss III); Perry *et*

*al.* (1981) *Infect. Immun. 32*:1295; Powell *et al.* (1988) *Appl. Environ. Microbiol. 54*:655; Somkuti *et al.* (1987) *Proc. 4th Evr. Cong. Biotechnology 1*:412, Streptococcus].

Yeast expression systems are also known to one of ordinary skill in the art. A yeast promoter is any DNA sequence capable of binding yeast RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site (the "TATA Box") and a transcription initiation site. A yeast promoter may also have a second domain called an upstream activator sequence (UAS), which, if present, is usually distal to the structural gene. The UAS permits regulated (inducible) expression. Constitutive expression occurs in the absence of a UAS. Regulated expression may be either positive or negative, thereby either enhancing or reducing transcription.

Yeast is a fermenting organism with an active metabolic pathway, therefore sequences encoding enzymes in the metabolic pathway provide particularly useful promoter sequences. Examples include alcohol dehydrogenase (ADH) (EPO Publ. No. 284 044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EPO Publ. No. 329 203). The yeast *PHO5* gene, encoding acid phosphatase, also provides useful promoter sequences [Myanohara *et al.* (1983) *Proc. Natl. Acad. Sci. USA 80*:1].

In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, UAS sequences of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (U.S. Patent Nos. 4,876,197 and 4,880,734). Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the *ADH2*, *GAL4*, *GAL10*, OR *PHO5* genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (EPO Publ. No. 164 556). Furthermore, a yeast promoter can include naturally

occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription. Examples of such promoters include, *inter alia*, [Cohen *et al.* (1980) *Proc. Natl. Acad. Sci. USA 77*:1078; Henikoff *et al.* (1981) *Nature 283*:835; Hollenberg *et al.* (1981) *Curr. Topics Microbiol. Immunol. 96*:119; Hollenberg *et al.* (1979) "The Expression of Bacterial Antibiotic Resistance Genes in the Yeast Saccharomyces cerevisiae," in: *Plasmids of Medical, Environmental and Commercial Importance* (eds. K.N. Timmis and A. Puhler); Mercerau-Puigalon *et al.* (1980) *Gene 11*:163; Panthier *et al.* (1980) *Curr. Genet. 2*:109;].

A DNA molecule may be expressed intracellularly in yeast. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Fusion proteins provide an alternative for yeast expression systems, as well as in mammalian, baculovirus, and bacterial expression systems. Usually, a DNA sequence encoding the N-terminal portion of an endogenous yeast protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the yeast or human superoxide dismutase (SOD) gene, can be linked at the 5' terminus of a foreign gene and expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. See e.g., EPO Publ. No. 196 056. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (e.g. ubiquitin-specific processing protease) to cleave the ubiquitin from the foreign protein. Through this method, therefore, native foreign protein can be isolated (*eg.* WO88/024066).

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provide for secretion in yeast of the foreign protein. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a

signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene (EPO Publ. No. 012 873; JPO Publ. No. 62,096,086) and the A-factor gene (U.S. Patent No. 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, exist that also provide for secretion in yeast (EPO Publ. No. 060 057).

A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated alpha-factor leaders (usually about 25 to about 50 amino acid residues) (U.S. Patent Nos. 4,546,083 and 4,870,008; EPO Publ. No. 324 274). Additional leaders employing an alpha-factor leader fragment that provides for secretion include hybrid alpha-factor leaders made with a presequence of a first yeast, but a pro-region from a second yeast alphafactor. (See e.g., PCT Publ. No. WO 89/02463.)

Usually, transcription termination sequences recognised by yeast are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator sequence and other yeast-recognised termination sequences, such as those coding for glycolytic enzymes.

Usually, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a prokaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 [Botstein *et al.* (1979) *Gene 8*:17-24], pCl/1 [Brake *et al.* (1984) *Proc.*

*Natl. Acad. Sci USA 81*:4642-4646], and YRp17 [Stinchcomb *et al.* (1982) *J. Mol. Biol. 158*:157]. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at least about 20. Enter a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host. See e.g., Brake *et al.*, *supra*.

Alternatively, the expression constructs can be integrated into the yeast genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to result from recombinations between homologous DNA in the vector and the yeast chromosome [Orr-Weaver *et al.* (1983) *Methods in Enzymol. 101*:228-245]. An integrating vector may be directed to a specific locus in yeast by selecting the appropriate homologous sequence for inclusion in the vector. See Orr-Weaver *et al.*, *supra*. One or more expression construct may integrate, possibly affecting levels of recombinant protein produced [Rine *et al.* (1983) *Proc. Natl. Acad. Sci. USA 80*:6750]. The chromosomal sequences included in the vector can occur either as a single segment in the vector, which results in the integration of the entire vector, or two segments homologous to adjacent segments in the chromosome and flanking the expression construct in the vector, which can result in the stable integration of only the expression construct.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of yeast strains that have been transformed. Selectable markers may include biosynthetic genes that can be expressed in the yeast host, such as *ADE2*, *HIS4*, *LEU2*, *TRP1*, and *ALG7*, and the G418 resistance gene, which confer resistance in yeast cells to tunicamycin and G418, respectively. In addition, a suitable selectable marker may also provide yeast with the ability to grow in the presence of toxic compounds, such as metal. For example, the presence of *CUP1* allows yeast to grow in the presence of copper ions [Butt *et al.* (1987) *Microbiol, Rev. 51*:351].

Alternatively, some of the above described components can be put together into transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, *inter alia*, the following yeasts:Candida albicans [Kurtz, *et al.* (1986) *Mol. Cell. Biol. 6*:142], Candida maltosa [Kunze, *et al.* (1985) *J. Basic Microbiol. 25*:141]. Hansenula polymorpha [Gleeson, *et al.* (1986) *J. Gen. Microbiol. 132*:3459; Roggenkamp *et al.* (1986) *Mol. Gen. Genet. 202*:302], Kluyveromyces fragilis [Das, *et al.* (1984) *J. Bacteriol. 158*:1165], Kluyveromyces lactis [De Louvencourt *et al.* (1983) *J. Bacteriol. 154*:737; Van den Berg *et al.* (1990) *Bio/Technology 8*:135], Pichia guillerimondii [Kunze *et al.* (1985) *J. Basic Microbiol. 25*:141], Pichia pastoris [Cregg, *et al.* (1985) *Mol. Cell. Biol. 5*:3376; U.S. Patent Nos. 4,837,148 and 4,929,555], Saccharomyces cerevisiae [Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA 75*:1929; Ito *et al.* (1983) *J. Bacteriol. 153*:163], Schizosaccharomyces pombe [Beach and Nurse (1981) *Nature 300*:706], and Yarrowia lipolytica [Davidow, *et al.* (1985) *Curr. Genet. 10*:380471 Gaillardin, *et al.* (1985) *Curr. Genet. 10*:49].

Methods of introducing exogenous DNA into yeast hosts are well-known in the art, and usually include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations. Transformation procedures usually vary with the yeast species to be transformed. See e.g., [Kurtz *et al.* (1986) *Mol. Cell. Biol. 6*:142; Kunze *et al.* (1985) *J. Basic Microbiol. 25*:141; Candida]; [Gleeson *et al.* (1986) *J. Gen. Microbiol. 132*:3459; Roggenkamp *et al.* (1986) *Mol. Gen. Genet. 202*:302; Hansenula]; [Das *et al.* (1984) *J. Bacteriol. 158*:1165; De Louvencourt *et al.* (1983) *J. Bacteriol. 154*:1165; Van den Berg *et al.* (1990) *Bio/Technology 8*:135; Kluyveromyces]; [Cregg *et al.* (1985) *Mol. Cell. Biol. 5*:3376; Kunze *et al.* (1985) *J. Basic Microbiol. 25*:141; U.S. Patent Nos. 4,837,148 and 4,929,555; Pichia]; [Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA 75*;1929; Ito *et al.* (1983) *J. Bacteriol. 153*:163 Saccharomyces]; [Beach and Nurse (1981) *Nature 300*:706; Schizosaccharomyces]; [Davidow *et al.* (1985) *Curr. Genet. 10*:39; Gaillardin *et al.* (1985) *Curr. Genet. 10*:49; Yarrowia].

Methods for the isolation and purification of recombinant proteins will be well known to those of skill in the art and are summarised, for example in Sambrook *et al* (1989). Particularly in bacteria such as *E. coli*, the recombinant protein will form inclusion bodies within the bacterial cell, thus facilitating its preparation. If produced in inclusion bodies, the carrier protein may need to be refolded to its natural conformation. Methods for renaturing proteins to their natural folded state are well known in the art.

Species in which the carrier proteins of the present invention may be immunogenic and thus effective in eliciting an immune response include all mammals, especially humans. In most cases, it will be preferred that the carrier proteins of the present invention are active eliciting an immune response in humans. The population of humans that are in greatest need of protection from disease caused by encapsulated bacteria are infants of between approximately 3 months and 2 years of age. It is during this period that the infants generally do not receive protection from mothers' milk and do not yet possess a sufficiently well-developed immune system themselves to generate an immune response against polysaccharide antigens.

According to a further aspect of the present invention, there are also provided nucleic acid molecules encoding carrier proteins according to the first aspect of the invention. As will be apparent to the skilled artisan, such nucleic acid molecules will be designed using the genetic code so as to encode the epitope that is desired.

Additionally, in order to precisely tailor the exact properties of the encoded carrier proteins, the skilled artisan will appreciate that changes may be made at the nucleotide level from known epitope sequences, by addition, substitution, deletion or insertion of one or more nucleotides. Site-directed mutagenesis (SDM) is the method of preference used to generate mutated carrier proteins according to the present invention. There are many techniques of SDM now known to the skilled artisan, including oligonucleotide-directed mutagenesis using PCR as set out, for example by Sambrook *et al.*, (1989) or using commercially available kits.

Most carrier proteins produced by such techniques of mutagenesis will be less efficacious than wild type proteins. However, it may be that in a minority of cases, such

changes produce molecules with improved carrier protein function as desired, such as greater immunogenicity in a certain organism.

The nucleic acid molecules according to this aspect of the present invention may comprise DNA, RNA or cDNA and may additionally comprise nucleotide analogues in the coding sequence. Preferably, the nucleic acid molecules will comprise DNA.

A further aspect of the present invention provides a host cell containing a nucleic acid encoding a carrier protein. A still further aspect provides a method comprising introducing the encoding nucleic acid into a host cell or organism. Introduction of nucleic acid may employ any available technique. In eukaryotic cells, suitable techniques may include calcium phosphate transfection, DNA-dextran, electroporation, liposome-mediated transfection or transduction using retrovirus or other viruses such as vaccinia. In bacterial cells, suitable techniques may include calcium chloride transformation, electroporation or transfection using bacteriophage. Introduction of the nucleic acid may be followed by causing or allowing expression from the nucleic acid, for example by culturing host cells under conditions for allowing expression of the gene.

In one embodiment, the nucleic acid is integrated into the genome of the host cell. Integration may be promoted by the inclusion of sequences that promote recombination with the genome, in accordance with standard techniques (see Sambrook *et al.*, 1989).

According to a further embodiment of the present invention, there is provided a carrier protein comprising at least five CD4+ T-cell epitopes, conjugated to polysaccharide. By polysaccharide is meant any linear or branched polymer consisting of monosaccharide residues, usually linked by glycosidic linkages, and thus includes oligosaccharides. Preferably, the polysaccharide will contain between 2 and 50 monosaccharide unites, more preferably between 6 and 30 monosaccharide units.

The polysaccharide component may be based on or derived from polysaccharide components of the polysaccharide capsule from many Gram positive and Gram negative bacterial pathogens such as *H. influenzae*, *N. meningitidis* and *S. pneumoniae*. This capsule represents a major virulence factor that is important for nasopharyngeal colonisation and systemic invasion. Other bacteria from which polysaccharide

components may be conjugated to the carrier proteins of the present invention include *Staphylococcus aureus*, *Klebsiella*, *Pseudomonas*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Shigella dysenteriae*. Polysaccharide components suitable for use according to this aspect of the present invention include the Hib oligosaccharide, lipopolysaccharide from *Pseudomonas aeruginosa* (Seid and Sadoff, 1981), lipopolysaccharides from *Salmonella* (Konadu *et al.*, 1996) and the O-specific polysaccharide from *Shigella dysenteriae* (Chu *et al*, 1991). Other polysaccharide components suitable for use in accordance with the present invention will be well-known to those of skill in the art.

Fragments of bacterial capsular polysaccharide may be produced by any suitable method, such as by acid hydrolysis or ultrasonic irradiation (Szn *et al*, 1986). Other methods of preparation of the polysaccharide components will be well known to those of skill in the art.

The polysaccharide component of the conjugate vaccine should preferably be coupled to the carrier protein by a covalent linkage. A particularly preferred method of coupling polysaccharide and protein is by reductive amination. Other methods include: activation of the polysaccharide with cyanogen bromide followed by reaction with adipic acid dihydrazide (spacer) and by conjugation to carboxide groups of carrier protein using soluble carbodiimides (Shneerson *et al*, 1986); functionalisation of the carrier protein with adipic acid dihydrazide followed by coupling to cyanogen bromide activated polysaccharides (Dick *et al*, 1989); chemical modification of both the carrier protein and the polysaccharide followed by their coupling (Marburg *et al*, 1986; Marburg *et al*, 1987 and 1989). In some cases, polysaccharides containing carboxide groups such as group C meningococcal polysaccharides can be directly conjugated to proteins using soluble carbodiimides. Polysaccharides can also be activated using alternative agents such as CDAP (1-cyano-4-dimethylamino-pyrridinium tetrafluorborate) and then directly conjugated to the carrier protein (Konadu *et al*, 1996). Periodate-treated polysaccharides or oligosachrides can all be conjugated to proteins by means of reductive amination (Jennings and Lugowsky, 1982; Anderson, 1983; Insel, 1986). Alternatively, oligosaccharides obtained by acidic hydrolysis can be chemically derivatised by introducing into their reducing end groups an hydrocarbon spacer bearing

an active ester terminus; this activated oligosaccharide can be conjugated to the selected carrier protein (Costantino *et al*, 1992).

The polysaccharide molecule may be coupled to the carrier protein by a spacer molecule, such as adipic acid. This spacer molecule can be used to facilitate the coupling of protein to polysaccharide. After the coupling reaction has been performed, the conjugate may be purified by diafiltration or other known methods to remove unreacted protein or polysaccharide components.

According to a further aspect of the present invention there is provided a method of production of a carrier protein according to the first aspect of the present invention, comprising the steps of:

(a) constructing oligonucleotide molecules that encode peptide epitopes;

(b) annealing the oligonucleotide molecules to form duplexes;

(c) introducing the oligonucleotide duplexes into an expression vector so as to encode a fusion protein;

(d) introducing the expression vector into a bacterial host cell to allow expression of the fusion protein;

(e) isolating the fusion protein produced from a culture of said bacteria.

Optionally, this method may additionally comprise conjugating the carrier protein to a polysaccharide molecule.

Preferably, the bacterial host cell used in this method is an *E. coli* bacterial host cell.

According to the further aspect of the present invention, there is provided a composition comprising a carrier protein that contains at least five CD4+ T-Cell epitopes conjugated to a polysaccharide, in conjunction with a pharmaceutically acceptable excipient. Such a composition may be rationally designed so as to provide protection against disease caused by pathogenic bacteria such as *H. influenzae*, *S. pneumoniae*, *N. meningitidis*, *Staphylococcus aureus*, *Klebsiella*, *Pseudomonas* and *S. typhi* and accordingly, may be

used as a vaccine. Vaccines according to the invention may either be prophylactic (*ie.* to prevent infection) or therapeutic (*ie.* to treat disease after infection).

By pharmaceutically-acceptable excipient is meant any compound that does not itself induce the production of antibodies harmful to the individual receiving the composition. The excipient should be suitable for oral, subcutaneous, intramuscular, topical or intravenous administration. Suitable compounds are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes) and inactive virus particles. Such compounds are well known to those of skill in the art. Additionally, these compounds may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminium salts (alum), such as aluminium hydroxide, aluminium phosphate, aluminium sulphate, *etc*; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components), such as for example (a) MF59™ (WO 90/14837), containing 5% Squalene, 0.5% Tween™ 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE, although not required) formulated into submicron particles using a microfluidizer (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidised into a submicron emulsion or vortexed to generate a larger particle sise emulsion, and (c) Ribi™ adjuvant system (RAS), containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Freund's complete and incomplete adjuvants (CFA & IFA); (5) cytokines, such as interleukins (*eg.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (*eg.* IFNγ), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the efficacy of the composition. Alum and MF59™ are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), *etc.*

The immunogenic compositions (*eg.* the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvanticity effect, as discussed above.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the carrier protein, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (*eg.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally *eg.* by injection, either subcutaneously or intramuscularly. They may also be administered to mucosal surfaces (*eg.* oral or intranasal), or in the form of pulmonary formulations, suppositories, or transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

As an alternative to protein-based vaccines, DNA vaccination may be employed [*eg.* Robinson & Torres (1997) *Seminars in Immunology* 9:271-283; Donnelly *et al.* (1997) *Annu Rev Immunol* 15:617-648]. Accordingly, rather than comprise a peptide, oligopeptide, or polypeptide compound, the vaccines of the invention might comprise nucleic acid encoding these compounds.

According to a further aspect of the invention, there is provided a process for the formulation of an immunogenic composition comprising bringing a carrier protein according to the first aspect of the invention, conjugated to a polysaccharide, into association with a pharmaceutically-acceptable carrier, optionally with an adjuvant.

According to a still further aspect of the present invention, there is provided a method of vaccinating a mammal, preferably a human against a disease, comprising administering to the mammal a composition of carrier protein conjugated to polysaccharide, optionally with a pharmaceutically-acceptable carrier such as an adjuvant.

Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to the carrier proteins N6 and N10 conjugated to HIB capsular polysaccharide. It will be appreciated that modification of detail may be made without departing from the scope of the invention. All publications, patents, and patent applications cited herein are incorporated <u>in full</u> by reference.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of the construction of the N6 protein.

Figure 2 illustrates the N6 and N10 constructs and their respective DNA and amino acid sequences. The histidine tag, the flag peptide, the Fxa cutting site and the CD4+ T cell epitopes are underlined.

Figure 3 is a Coomassie-stained SDS-PAGE gel of total protein extracts prepared from induced *E. coli* clones producing the different polyepitope proteins. Lane A: negative control (TG1 cells containing pQE30 vector with no insert); lane B: TG1 cells containing the pQE30-N10 plasmid; lane C: TOP10 cells containing the pTrc-N10

plasmid; lane D: TOP10 cells containing the pTrc-N6 plasmid; lane E: low molecular weight markers.

Figure 4 is an immunoblot of the SDS-PAGE gel that is illustrated in Figure 3. The Western blot was incubated with a rabbit antiserum specific for the flag peptide and then with a peroxidated anti-rabbit IgG antibody. The immune reaction was then revealed using 4-chloro-1-napthol as substrate for the peroxidase.

Figure 5 is an SDS-PAGE Coomassie-stained gel containing different samples obtained during the procedure of purification of the N6 protein. Lane A: starting material (total protein of the induced TOP10 *E. coli* cells containing pTrc-N6 plasmid; lane B: soluble proteins (supernatant obtained after centrifugation of the total protein sample); lane C: proteins soluble in 1M urea (supernatant obtained after washing the insoluble proteins with 1M urea); lane D: inclusion bodies (pellet obtained after washing the insoluble proteins with 1M urea); lane E: N6 protein obtained from purification on $Ni^{2+}$ NTA resin using the immobilised metal affinity chromatography (IMAC) technique; lane F: low molecular weight markers.

Figure 6 is an immunoblot of the SDS-PAGE gel that is illustrated in Figure 5. The Western blot was incubated with a rabbit antiserum specific for the flag peptide and then with a peroxidated anti-rabbit IgG antibody. The immune reaction was then revealed using 4-chloro-1-napthol as substrate for the peroxidase.

Figure 7 is a schematic representation of N11 construct and its respective DNA and protein sequence. The hexahistidine tag, the flag peptide, the FXa cutting site, and the CD4+ T cell epitopes are underlined.

Figure 8 is a schematic representation of N19 construct and its respective DNA and protein sequence. The hexahistidine tag, the flag peptide, the FXa cutting site, and the CD4+ T cell epitopes are underlined.

Figure 9 is an SDS-Page and Coomassie staining of proteins coming from Top10-Trc-N11 *E. coli* clone.

Lane A: Total extract of an uninduced culture.

Lane B: Total extract of a culture induced using IPTG.

Lane C: purified N11 protein (solubilisation of whole cells with guanidinium and IMAC chromatography).

Figure 10:

A: SDS-Page and Coomassie staining. Analysis of the fractions obtained from IMAC chromatography performed to purify N19 protein. Lane a: prestained molecular weight markers. Lane b: flow through. Lanes from c to m: gradient fractions showing the purified N19 protein; the bands having a molecular weight lower than N19 and visible in the overloaded lanes f, g, and h represents degradation products of the N19 protein.

B: SDS-Page and Coomassie staining. Analysis of the fractions obtained from IMAC chromatography of the N19 conjugated to Hib polysaccharide. All N19 protein resulted to be conjugated, as judged by the high molecular weight of the conjugate and by the absence of 43.000 kDa unconjugated N19 protein.

C: The same conjugate samples used in picture B were subjected to western immuno-blot using an anti-flag antibody. Also here it can be appreciated that all N19 protein migrated as a very high molecular weight after conjugation to Hib polysaccharide, and that there is not unconjugated N19 protein migrating at 43.000 kDa.

Figure 11: Proliferative response of two human T cell clones specific for P30TT (GG-22 clone) and P2TT (KSIMK-140 clone) after stimulation with the respective synthetic peptides (controls) and with conjugated or nunconjugated polyepitope proteins (cpm: counts per minute).

Figure 12: Peripheral blood mononuclear cells (PBMC) proliferation asssay. PBMC from three healthy donors, RR, EB and MC, immune to tetanus toxoid were stimulated with tetanus toxoid, P2TT, N6, N6-Hib and N10-Hib.

Figure 13: Results of the immunogenicity tests performed to compare the carrier effect of N10, N19, and CRM-197, and to check for carrier induced immunosuppression phenomena. Anti-Hib titres obtained after immunising primed and unprimed CD1 mice with different conjugates.

## DETAILED DESCRIPTION OF THE INVENTION

## MATERIALS AND METHODS

### Summary of standard procedures and techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature *eg.* Sambrook *Molecular Cloning; A Laboratory Manual, Second Edition* (1989); *DNA Cloning, Volumes I and ii* (D.N Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed, 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. 1984); *Transcription and Translation* (B.D. Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.I. Freshney ed. 1986); *Immobilised Cells and Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide to Molecular Cloning* (1984); the *Methods in Enzymology* series (Academic Press, Inc.), especially volumes 154 & 155; *Gene Transfer Vectors for Mammalian Cells* (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory); Mayer and Walker, eds. (1987), *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London); Scopes, (1987) *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), and *Handbook of Experimental Immunology, Volumes I-IV* (D.M. Weir and C. C. Blackwell eds 1986).

### Plasmids, strains and T cell clones.

PEMBLex2 plasmid was derived from pEMBL8M (Dente L. and Cortese R., *Meth. Enzymol.* (1987), **155**: 111-9) by inserting a $\lambda P_L$ promoter and a polylinker into the *Eco*RI and *Hind*III sites. The commercial vectors pTrc-His and pQE30 were purchased from Invitrogen and Qiagen respectively. *E. coli* strains used as recipients of the above plasmids were: K12$\Delta$H1$\Delta$Trp for pEMBLex2, TOP10 for pTrc-His and TG1 for pQE30.

Human T cell clones KSMIK 140 and GG-22 specific for P2TT and P30TT respectively were kindly provided by Dr. A. Lanzavecchia (Basel, Switzerland).

**Construction of recombinant plasmids that express the N6 polyepitope carrier protein.**

Complementary oligodeoxyribonucleotide pairs coding for P2TT, P21TT, P23TT, P30TT1, P32TT and PfT3 T cell epitopes (Table 1) and for a Flag peptide were synthesised using the DNA synthesiser ABI394 (Perkin Elmer) and the reagents from Cruachem (Glasgow, Scotland). The oligo pairs were separately annealed in T4 DNA ligase buffer (Boehringer Mannheim) and equimolar amounts of each annealing reaction were mixed and ligated at room temperature for 3 hours using T4 DNA ligase (Boehringer Manheim).

The ligase reaction was then loaded onto a 1% agarose gel and subjected to electrophoresis. The bands corresponding to the DNA fragments of expected size were isolated, purified and cloned into the pEMBLex2 expression vector using standard protocols (Sambrook *et al.*, 1989). After transformation, a rabbit antiserum specific for the Flag peptide was used to perform colony-screenings (Sambrook *et al.*, 1989) in order to identify recombinant protein producing clones. Protein extracts from positive clones were analysed using SDS-PAGE to further select for clones on the basis of recombinant protein size.

| T cell epitope | Aminoacid position | Aminoacid sequence | References |
|---|---|---|---|
| P23TT | 1084-1099 | VSIDKFRIFCKANPK | Demotz et al. 1993 *Eur.J Immunol* 23: 425 |
| P32TT | 1174-1189 | LKFIIKRYTPNNEIDS | Demotz et al. 1993 *Eur J Immunol* 23: 425 |
| P21TT | 1064-1079 | IREDNNITLKLDRCNN | Dr. Lanzavecchia, pers. comrn. |
| PF T3 | 380-398 | EKKIAKMEKASSVFNVVN | Hammer et al. 1993 *Cell* 74: l97 |
| P30TT | 947-967 | FNNFTVSFWLRVPKVSASHLE | Demotz et al. 1993 *Eur.J Immunol* 23: 425 |
| P2TT | 830-843 | QYIKANSKFIGITE | Demotz et al. 1993 *Eur.J Immunol* 23: 425 |
| HA | 307-319 | PKYVKQNTLKLAT | Alexander et al. 1994 *Immunity* 1: 751 |
| HBVnc | 50-69 | PHHTALRQAILCWGELMTLA | Alexander et al. 1994 *Immunity* 1: 751 |
| HBsAg | 19-33 | FFLLTRILTIPQSLD | Greenstein et al. 192 *J Immunol* 148: 3970 |
| MT | 17-31 | YSGPLKAEIAQRLEDV | Alexander et al. 1994 *Immunity* 1: 751 |
| HSP70 | 408-427 | QPSVQIQVYQGEREIASHNK | Adams et al. 1997 Infect Immun 65: 1061 |
| Flag peptide | | MDYKDDDD | |

**Table I.** CD4+ T cell epitopes inserted in the recombinant polyepitope carrier proteins.

After nucleotide sequencing of the selected clones, a clone named pEMBLN6 was shown to contain six different T cell epitopes with no repetitive sequences. The N6 insert was then PCR-amplified and transferred to pTrc-His expression vector (Invitrogen) using standard techniques (Sambrook *et al.*, 1989). The generation of the N6 expressing plasmids is summarised in Figure 1.

**Construction of recombinant plasmids that express the N10 polyepitope carrier protein**

Using synthetic oligodeoxyribonucleotides and standard cloning techniques (Sambrook *et al.*, 1989), four additional CD4+ T cell epitopes were added to the N6 protein: HBVnc, HA, HbsAg, and MT (Table I). HBVnc and HA were sequentially introduced into pTrc-N6 by means of two consecutive cloning steps; to the resulting plasmid the HbsAg and MT epitopes were added in a single cloning step.

After DNA sequencing, a correct construct (pTrc-N10) coding for the expected ten epitope polyepitope protein was identified. The N10 coding insert was then transferred from pTrc-N10 to pQE30 (Qiagen) by means of PCR. The sequence of the resulting pQE-N10 construct was then confirmed by DNA sequencing.

**Construction of the recombinant plasmid expressing N11 polyepitope carrier protein.**

Two complementary oligodeoxyribonucleotides were synthesised and annealed to obtain a DNA linker coding for the HSP70 CD4+ T cell epitope (Table I). The linker was inserted in pTrc-N10 plasmid downstream from N10 coding region and in frame with it. After transformation in TOP10 *E. coli* strain, the transformants were selected using protein expression and DNA sequencing analyses. Glycerol batches of a selected clone (TOP10/pTrc-N11) having the correct coding sequence and expressing a protein of the expected molecular weight were stored to -80°C.

**Construction of recombinant plasmids that express the N19 polyepitope carrier protein.**

The DNA fragment encompassing the coding region from P23TT to HBsAg was PCR amplified using the plasmid pTrc-N10 as template and two oligonucleotide primers which allow the insertion of BglII and PstI restriction sites respectively at the 5' and 3' ends of the PCR product. The plasmid pTrc-N10 was digested with BamHI and PstI restriction enzymes and ligated to the PCR product digested with BglII and PstI. After transformation in TOP10 cells and selection of the transformants using protein expression and DNA sequencing analyses, glycerol batches of a selected clone (TOP10/pTrc-N19) having the correct coding sequence and expressing a protein of the expected molecular weight were stored to -80°C.

The pTrc-N19 plasmid was digested with EcoRV and PstI and the insert was cloned in pQE-N10 digested with the same enzymes. After transformation in TG1 cells and selection of the transformants using protein expression and DNA sequencing analyses, glycerol batches of a selected clone (TG1/pQE-N19) having the correct coding sequence and expressing a protein of the expected molecular weight were stored to -80°C.

**Purification of the polyepitope carrier proteins.**

All the recombinant polyepitope carrier proteins were purified using a similar strategy. Briefly, *E. coli* cultures were grown in 500 ml LB medium containing 100 μg/ml Ampicillin, at 37 $^0$C. At 0.3-0.5 $OD_{600}$ , the expression of the polyepitope proteins was induced for 3-5 hours by adding 0.1-1 mM IPTG. Cells were disrupted by sonication or French press, the insoluble fraction was collected by centrifiagation, dissolved with buffer A (6 M guanidiniwn-HCL, 100 mM $NaH_2PO_4$, 10 mM Tris base, pH 8) and adsorbed with 2 ml of $Ni^{2+}$NTA resin (Qiagen).

Then, the resin was packed in a column and washed with buffer A. Guanidinium-HCI was removed from the column by washing with buffer B (8 M Urea, 100 mM $NaH_2PO_4$, 10 mM Tris base) pH 8. After a wash with buffer B pH 6.5, recombinant proteins were eluted with a 20 ml buffer B gradient from ph 6.5 to pH 4. The factions containing the purified recombinant proteins were pooled and dialysed against PBS, pH 7.2. Proteins were analysed by SDS_PAGE and protein content was determined using the Bradford method. Alternatively, cell pellets obtained from *E coli* cultures were solubilized by heating at 37 °C in buffer A, the lysates were centrifuged to 15.000 g for 20 min. The

supernatants were subjected to column chromatography on Nickel activated Chelating Sepharose Fast Flow (Pharmacia). After a wash with buffer A and a wash with buffer B, pH 7, the proteins were separated by collecting fractions from a 0-200 mM gradient of Imidazole in buffer B, pH 7. The fractions containing the purified recombinant proteins (as judged by SDS-PAGE and Coomassie staining) were pooled and dialysed against PBS, pH 7.2.

**Preparation and activation of Hib oligosaccharides.**

The Hib capsular polysaccharide can be prepared according to the protocol described in Gotschlich *et al.* (1981) *J. Biol. Chem.* **256**: 8915-8921.

1.99L of a 10 mg/ml solution of Hib polysaccharide was hydrolysed in 0.01M acetic acid at 76°C for 5 hours. After chilling, neutralization and 0.2μm filtration, the resulting oligosaccharide population had an average degree of polymerisation (avDP) of 8 as measured by the chemical ratio between ribose and reducing groups.

NaCl was then added to the hydrolysate until a concentration of 0.16 M was attained, then diluted 1:1 with 0.16M NaCl/ 10mM acetate pH 6 and submitted to tangential flow ultrafiltration on a 10 kDa membrane in order to remove high molecular weight species.

Ultrafiltration comprised approximately 11-fold concentration followed by 15 cycles of diafiltration against 0.16 M NaCl/ 10mM acetate, pH 6. The retentate was discarded. The permeate was diluted :1 with water and 0.22 μm filtered. Chemical analysis revealed an avDp of 8.1.

The permeate obtained from 10 kDa UF was loaded, at a linear flow rate of 150 cm/h, onto a Q-Sepharose Fast Flow column [10 cm (ID); 5,5 cm (h)] equilibrated with 0.08 M NaCl/0.05 M sodium acetate pH 6. After adsorption, low molecular weight fragments (up to 5 repeats) were removed by washing the column with 10 column volumes of equilibrating buffer and then eluted with 3 column volumes of 0.5 M NaCl/0.005M sodium acetate pH 6. The eluate was 0.2 μm filtered and then analysed for avDp and ion exchange analytical chromatography. AvDP resulted at 17.3, ion exchange analytical chromatography on Mono Q HR 5/5 revealed the absence of any small fragments until DP 5.

To introduce a terminal amino group, reductive amination was then performed; to the fractionated Hib oligosaccharide obtained from Q-Sepharose chromatography, ammonium chloride 35mg/ml and sodium cyanoboroidride 12 mg/ml final concentrations were added. After stirring, the solution was 0.2μm filtered and incubated at 37°C for 120 hours. The amino oligosaccharide was then purified from excess of reagents by precipitation with 95° EtOH (81° final concentration) in the cold for 15-20 hours. The precipitated oligosaccharide was then recovered by centrifugation, solubilized in NaCl 0.4M using approximately 1/4 of the starting volume and precipitated again at 81° EtOH in the cold for 15-20 hours.

The amino-oligosaccharide was again recovered by centrifugation and solubilized in about 300 ml of 0.02 M NaCl. After having taken a sample for analysis, the resulting solution was then dried using a rotary evaporator.

Colorimetric amino group analysis confirmed the introduction of a primary amino group into the oligosaccharide.

Derivatisation to active ester was then performed as follows. The amino-oligosaccharide was solubilised in distilled water at a concentration of 40μmol of amino groups per ml. The solution was then diluted 10-fold with DMSO. Triethylamine was added in molar ratio to the amino groups of 2:1. N-hydroxysuccinimido diester of adipic acid was then added in a molar ratio to the amino groups of 12:1. The reaction mixture was kept under gentle stirring for 2 hours at RT. The activated oligosaccharide was then purified from the excess of reagents by precipitation into 10 volumes of 1-4 dioxane under stirring. After 30 minutes in the cold the precipitate was collected onto a syntered glass filter, washed onto the filter with dioxane and then dried under vacuum. The dried activated oligosaccharide was analysed for its content of active ester groups by a colorimetric method; this test showed the presence of 62.1μmol of active ester per mg of dried oligosaccharide.

The above-obtained activated oligosaccharide was then used for conjugation experiments.

**Conjugation of the polyepitope carrier protein with Hib capsular oligosaccharides and purification of the conjugates.**

33.4nmoles of recombinant carrier protein and 669nmoles of activated Hib oligosaccharide in a final volume of 0.5 ml 10mM phosphate buffer, pH 7, were gently stirred overnight at RT and brought up to 5ml 1M $(NH_4)_2SO_4$, 10mM phosphate pH7. The sample was subjected to FPLC on a 1ml Phenyl Sepharose 5/5 HR column (Pharmacia). 1ml fractions were collected both during washing (1M $(NH_4)_2SO_4$, 10mM phosphate, pH 7) and elution (10mM phosphate, pH 7). Two peaks corresponding to the non-adsorbed material and to the eluted material were obtained. The pooled fractions corresponding to the non-adsorbed material and the pooled fractions corresponding to the elution peak were subjected to protein and ribose content determination and to SDS-PAGE and Western blot analysis.

A protocol to conjugate recombinant proteins to oligosaccharides directly on $Ni^{2+}$–NTA resin was also developed. Recombinant proteins were purified as described above, but the final dialysis step was omitted. The protein content of the 8M urea fraction pool was measured with the Bradford assay. The pH of the eluted proteins was adjusted to pH8 and adsorption on 1ml pre-equilibrated $Ni^{2+}$–NTA resin was again performed in a batch mode. Urea was removed by washing with 4x25ml 100mM phosphate buffer pH 7.5. The resin was suspended in 1ml 100mM phosphate buffer pH 7.5 and a 20-fold molar excess of activated Hib oligosaccharide (as compared to the protein that was adsorbed on the resin) was added to the suspension. The mixture was gently stirred overnight at RT packed in a column, and washed with 50ml 100mM phosphate buffer pH 7.5 to remove unconjugated oligosaccharide.

Elution of the conjugate was performed with 100mM phosphate buffer pH 4. Peak fractions were pooled and dialysed against PBS, pH 7.2. The conjugate was analysed by Coomassie staining of SDS-PAGE gels and Western immunoblot using an anti-flag rabbit antibody. The protein/carbohydrate ratio of the glycoconjugate was determined upon Bradford assay and ribose content determination.

**Cultures of PBMCs and T cell clones.**

Culture medium for PBMCs was RPMI 1640 (Gibco Laboratories, Paisley, Scotland) supplemented with 2mM L-glutamine, 1% nonessential amino acids, 1mMsodium pyruvate, gentamycin (50μg/ml), and 5% human serum (RPMI-HS) or 10% foetal calf serum (RPMI-FCS). For the growth of T-cell lines and clones, RPMI-HS was

supplemented with 50U of recombinant interleukin-2 (rIL-2: Hoffmann La Roche, Nutley, NJ) per ml.

**PBMC Proliferation Assay.**

Frozen PBMC ($10^5$) from healthy adults immune to tetanus toxoid were thawed and cultured in duplicate wells of 96-well flat-bottomed microplates, in 0.2ml of RPMI-HS (Di Tommaso *et al*, 1997). The recombinant proteins and tetanus toxoids (Chiron, Siena) were added to wells at the final concentration of 10μg/ml. After 5 days of culture. 1μCi of [$^3$H] thymidine (specific activity: 5Ci/mmol, Amersham) was added to each well and DNA-incorporated radioactivity was measured after an additional 16hrs by liquid scintillation counting.

**Proliferation assay of T cell clones.**

Two Human T cell clones, KSMIK 140, and GG-22, specific for P2TT and P30TT respectively, and the respective peptides were kindly provided by Dr. A. Lanzavecchia (Basel, Switzerland). T cells (2 x $10^4$) were cultured with autologous irradiated Epstein Barr Virus-transformed B lymphocytes (3 x $10^4$) in 0.2 ml of RPMI-FCS in 96-well flat-bottomed microplates in duplicate wells. Synthetic peptides and conjugated or unconjugated recombinant proteins were added to cultures at a final concentration of 10μg/ml. After 2 days, 1 μCi of [$^3$H]thymidine was added and the radioactivity incorporated was measured by liquid scintillation counting after an additional 16 hours.

In some experiments, carrier proteins and their conjugates were pre-incubated with APCs for 2-4 hours, then APCs were washed and cultured with T cell clones. This procedure was used to limit possible proteolytic degradation by serum proteases and to be more confident that epitope presentation would be due to intracellularly-processed epitopes.

**Immunogenicity tests.**

In a first experiment, equal doses of the glycoconjugates and of the polysaccharide (2.5μg as polysaccharide) in presence of 0.5mg of aluminium hydroxide as adjuvant were injected subcutaneously into groups of eight BALB/c and C57BL/6 mice (female, 7-week-old) on days 0, 21 and 35. Mice were bled on day –1 (pre-immune), 20 (pre-2),

34 (pre-3) and 45 (post-3) and individual sera collected and stored at –80°C before ELISA assay.

In a second experiment, equal doses of the glycoconjugates and of the polysaccharide (2.5μg as polysaccharide) in the presence of 0.5mg of aluminium hydroxide as adjuvant were injected subcutaneously into groups of eight Swiss ('D1 and BALB/c mice (female, 7-week-old) on days 0, 10 and 20. A boost of 2.5μg of purified Hib polysaccharide (HibCPS) in presence of 0.5 mg of aluminium hydroxide was then given to each mouse at day 70. Mice were bled on day –1 (pre-immune). 35 (post-vaccination), 68 (pre-boost) and 85 (post-boost) and individual sera collected and stored at –80°C before ELISA assay.

In a third experiment, equal doses of CRM-Hib, N10-Hib, and N19-Hib (2.5 μg as polysaccharide) in presence of 0.5 mg of aluminium hydroxide as adjuvant were injected subcutaneously in groups of 6 Swiss CD1 mice (female, 7-week-old) on days 0, 15, and 28 in order to compare the carrier effects. Different groups of mice were also subjected to the same schedule but were previously primed with unconjugated carriers in order to check for potential immunosuppression phenomena. In the latter groups equal doses of carrier proteins (50 μg) in 0.5 mg alum were injected on day -30. All mice were bled on day -32 (pre-priming), -2 (pre-immune), 14 (post-1), 27 (post-2), and 45 (post-3) and the sera were collected and stored to -80°C before ELISA assay.

**ELISA.**

Nunc Maxisorp 96-well flat-bottomed plates were coated by overnight incubation at 4°C with 1μg/ml (as polysaccharide) of a human serum albumin (HSA) and *H. influenzae* type b polysaccharide conjugate (HSA-Hib). After washing, wells were over-coated using 1% (w/v) gelatin in PBS, pH 7.2 for 3 additional hours at 37°C. Serum samples were diluted 1:50 in 5mM phosphate buffer, pH 7.2 containing 75mM NaCL 1% (w/v) BSA and 0.05% (w/v) Tween-20 and dispensed in duplicate into the wells. Sera from untreated mice were pooled and diluted 1:50 as above and dispensed into 8 wells. After overnight incubation at 4°C, plates were washed three times with 5mM phosphate buffer, pH 7.2 containing 75mM NaC1 and 0.05% (w/v) Tween-20. Then, alkaline phosphate-conjugated goat 1gG anti-mouse 1gG diluted 1:1000 and 5mM phosphate

buffer, pH 7.2 containing 75mM NaC1. 1% (w/v) BSA and 0.05% (w/v) Tween-20 were added to each well, and incubated 3 hours at 37°C.

After repeated washing, 100µl of a chromogen-substrate, *p*-nitrophenylphosphate, in a diethylenamine solution, were added to each well. Reaction was stopped after 20min by adding a 4N NaOH solution. Then, the plate was read at 405mM with a reference wavelength of 595mM. Titres were expressed as absorbencies at 405mM ($A_{405mm}$). Mice were considered responders when the average $A_{405mm}$ was found equal to or higher than four times the average of absorbencies of the eight wells with the sera from untreated animals. According to the European Pharmacopoeia [PA/PH/Exp15/T(93)3ANP] four out of eight mice should be responders.

In the second experiment, mice were considered responders when the average $A_{405mm}$ was found four times the average of the absorbencies of eight pre-immune sera of the same group of treatment.

The anti-carrier response was assayed as above described for anti-Hib response using plates coated with N10 or N6 (coating concentration = 2µg/ml).

## RESULTS

### Construction of the Polyepitope Carrier Proteins.

Using the approaches described in materials and methods, we created several *E. coli* clones expressing different carrier proteins. The following table lists only the six clones we utilised to purify the recombinant polyepitope carrier proteins:

| Name of the clone | Expressed polyepitope protein | Number of aminoacids | Theoretic Mol. W. (kDa) | E. coli host strain | Expression vector |
|---|---|---|---|---|---|
| Top10-Trc-N6 | N6 | 143 | 16 | Top10 | pTrc-His |
| Top10-Trc-N10 | N10 | 218 | 24 | Top10 | pTrc-His |
| TG1-QE-N10 | N10 | 218 | 24 | TG1 | pQE30 |
| Top10-Trc-N11 | N11 | 240 | 27 | Top10 | pTrc-His |
| Top10-Trc-N19 | N19 | 390 | 43 | Top10 | pTrc-His |
| TG1-QE-N19 | N19 | 390 | 43 | TG1 | pQE30 |

The clone expressing N6 protein comprised the plasmid pTrc-N6 transformed in the Top10 *E. coli* strain. As deduced from plasmid DNA sequencing, this plasmid code for a protein having an hexahistidine amino terminal tail followed in sequence by a flag peptide, a FXa site, and the following T cell epitopes: P23TT, P32TT, P21TT, PfT3, P30TT, and P2TT. All the epitopes were spaced by a KG aminoacid sequence (Fig.2).

The two clones that produced N10 protein were the Top10 *E. coli* strain containing the plasmid pTrc-N10, and the TG1 *E. coli* strain containing the plasmid pQE-N10. Both these clones contained the N6 coding sequence fused to a carboxy terminal sequence coding for four additional T cell epitopes which were in the order: HBVnc, HA, HBsAg, and MT (Fig 2).

The clone that produced N11 protein comprised the plasmid pTrc-N10 transformed in the Top10 *E. coli* strain. As deduced from plasmid DNA sequencing, this plasmid code for a protein consisting in the N10 sequence fused to a carboxy terminal sequence coding for the HSP70 T cell epitope (Fig. 7).

The two clones that produced N19 protein were the Top10 *E. coli* strain containing the plasmid pTrc-N19, and the TG1 *E. coli* strain containing the plasmid pQE-N19. Both these clones contained the N10 coding sequence fused to a carboxy terminal sequence coding for nine additional T cell epitopes which were in the order: P23TT,

P32TT,P21TT, PfT3, P30TT, P2TT, HBVnc, HA, and HBsAg (Fig 8).

**Protein Expression and Purification.**

Figures 3 and 4 depict protein expression of the three synthetic proteins. The addition of four new epitopes (HBVnc, HA, HbsAg, and MT) to N6 in pTrc-His (lane D) to obtain N10 protein (lane C) resulted in a remarkable reduction of protein expression. An attempt to increase the expression level of N10 simply involved changing the expression vector (from pTrc)-His to pQE30) and the *E. coli* strain (from Top10 to TG1). As seen in Figures 3 and 4, the amount of N10 expressed by pQE30-N10 in TG1 (lane B) was notably higher than the same protein expressed by pTrc-N10 (lane C). This is thought possibly to be due to the fact that whereas N6 protein was effectively assembled by the *E. coli* strain in the order of epitopes most suited to the organism, whereas the addition of four further epitopes was effectively forced and thus was less natural. However, the fact that the level of N10 expression was notably increased by simply changing expression vector (from pTrc-His to PQE30) and *E. coli* strain (from TOP-10 to TG1) suggests that additional factors, other than epitope combination, play a role in protein expression.

Figure 9 shows protein expression and purification of the N11 protein (SDS-PAGE and Coomassie staining). Total extract coming from an induced culture (lane B) shows an induced band, corresponding roughly to the expected molecular weight of N11 protein, that is not present in uninduced extract (lane A). The identity of the induced band was established also by western blot using an anti-flag antibody, and was also deduced from plasmid DNA sequencing (figure 7). N11 purification (figure 9, lane C) was done by solubilising whole cell pellets in guanidinium and by subjecting the whole extract to IMAC chromatography, with this procedure we obtained 14 mg of recombinant N11 protein from one litre of Top10-Trc-N11 flask culture. The addition of HSP70 T cell epitope to the carboxy terminus of N10 resulted in a construct (pTrc-N11) that was able to notably improve the expression of the polyepitope protein as compared to the expression obtained from pTrc-N10.

As it was for the N10 protein, also the expression of N19 protein was improved by changing the expression vector (from pTrc-His to pQE30) and the host strain (from Top10 to TG1). TG1(QE-N19) was used to purify N19 polyepitope protein. By

subjecting solubilised inclusion bodies to IMAC chromatography, we purified (see figure 10A) 5.42 mg of N19 protein from one litre of flask culture. The identity of N19 was identified in SDS-Page as an induced band having the expected molecular weight, in immuno western blot using an anti-flag antibody, and was also deduced after plasmid DNA sequencing (figure 8).

All clones expressing recombinant polyepitope proteins produced them mainly in the form of inclusion bodies. Purification of N6 and N10 proteins from inclusion bodies solubilised with 8M urea using an immobilised metal affinity chromatography (IMAC) procedure in the presence of 8M urea resulted in the loss of a high percentage of protein which was elutable with a 6.5-4 pH gradient (data not shown).

On the contrary, almost all of the histidine-tagged protein was eluted with the 6.5-4 pH gradient when starting inclusion bodies were solubilised with 6M guanidine hydrochloride (Figures 5 and 6). Using this protocol 7.8mg of N6 was purified from a litre of culture. The N10 protein that was employed in immunisation and T cell proliferation experiments was purified from pTrc-N10 clone.

Given the lower expression of recombinant protein shown by this clone we decided to purify N10 protein by solubilising whole cells with guanidinium in such a way as to exploit soluble and insoluble (inclusion bodies) proteins for IMAC purification. With this procedure 1.5 mg of purified N10 protein was obtained from a litre of culture. The higher success of solubilisation using 6M guanidium is thought to be due to the ability of this compound to solubilise the carrier proteins in monomeric form.

**Hib oligosaccharide conjugation to polyepitope proteins.**

Using the phenyl sepharose FPLC protocol we obtained a purified N6-Hib conjugate having a protein content of 79.4 μg/ml, and an oligosaccharide content of 42.7μg/ml.

We observed that 30% of conjugated protein was unable to bind to phenyl sepharose in the presence of 1M $(NH_4)_2SO_4$. In addition, 30-40% of carrier protein was previously lost during a dialysis step to remove urea before the conjugation reaction. To overcome these problems it was checked if it was possible to perform the conjugation reactions when the protein was adsorbed on the $Ni^{2+}$-NTA resin. We observed that the Hib

oligosaccharide was unable to bind $Ni^{2+}$-NTA resin at any pH, suggesting the feasibility of this approach and predicting that no interference due to the oligosaccharide could influence the elution of the protein once conjugation had taken place.

A reaction was thus set up involving protein adsorption on $Ni^{2+}$-NTA resin in the presence of 8M urea, urea removal, conjugation with oligosaccharide, washing, and conjugate elution. No aggregation phenomena were observed for the eluted conjugate. Using this procedure we obtained a purified N6-Hib conjugate having a protein content of 320μg/ml and an oligosaccharide content of 370μg/ml. and a purified N10-Hib having a protein content of 113μg/ml and an oligosaccharide content of 114μg/ml.

By using a 1:10 protein to carbohydrate molar ratio to conjugate oligosaccharide to recombinant carriers, we observed that a fraction of protein remained unconjugated (as judged by Coomassie staining of SDS-PAGE gel and Western immunoblot; data not shown). When a 1:20 protein to carbohydrate stoichiometric ratio was used, all the purified recombinant proteins were found to be completely conjugated, in fact, by analysing Coomassie-stained gels and western immunoblots using an anti-Flag antibody. We observed that after conjugation of N6 and N10 with Hib oligosaccharides these molecules increased their molecular weight, appearing as a high molecular weight smear, and proteins were no longer visible at the expected molecular weight for N6 and N10 monomers. This suggested that the synthetic proteins were completely conjugated to Hib oligosaccharides (data not shown).

The conjugation of activated Hib oligosaccharide to N19 protein resulted in a protein content of 173 μg/ml and in an oligosaccharide content of 127 μg/ml. Figure 10B depicts an SDS-Page and Coomassie staining analysis of the fractions obtained from IMAC chromatography of the N19 conjugated to Hib polysaccharide. All N19 protein resulted to be conjugated, as judged by the high molecular weight of the conjugate and by the absence of 43.000 kDa unconjugated N19 protein. Figure 10C shows the corresponding western immuno-blot using an anti-flag antibody. Also here it can be appreciated that all N19 protein migrated as a very high molecular weight after conjugation to Hib polysaccharide, and that there is not unconjugated N19 protein migrating at 43.000 kDa.

**Recognition of carrier proteins and their conjugates by human T lymphocytes.**

To investigate whether T cell epitopes contained in the polypeptides were recognised by human T cells we used T cell clones specific for the TT universal epitopes p2TT and p30TT (Demotz *et al.* 1993). Figure 11 shows that N6 is recognised by both clones not only as a simple polypeptide but also after it has been conjugated with polysaccharide. Remarkably, N6-Hib is recognised even better than unconjugated N6 by the T cell clone specific for P2TT. N10-Hib is recognised by the clone specific for p2TT but is poorly recognised by the clone specific for P30TT. In both cases N10-Hib exerts the same stimulatory activity as the synthetic peptide. The N10 clone was not tested in these experiments.

Once assessed that the T cell epitopes contained in the carrier proteins are correctly presented to T lymphocytes, we asked whether these carriers maintain their stimulatory capacity when presented to a heterogeneous population of lymphocytes such as PBMC. This could be predictive of whether our carriers might function as such once injected into subjects immune to antigens whose epitopes are included in the carriers themselves. For this purpose we used PBMC from donors immune to TT (A. Di Tommaso *et al.* 1997), since TT epitopes are the most represented in our polypeptides. Figure 12 shows that all the formulations were able to stimulate PBMC proliferation.

However, the incubation of PBMC with a synthetic peptide representing one of the epitopes included in both N6 and N10 constructs failed to exert a stimulatory effect. As a positive control, the PBMC were also incubated with 10 μg/ml of TT, that in all cases induced a proliferative response. Interestingly, the N6 polyepitope protein turned out to be the most potent PBMC stimulator among those tested in two out of three volunteers.

**Immunogenicity Tests.**

The carrier effect of the proteins N10 and N6 in comparison with CRM197 was assayed in mice in several glycoconjugate vaccines. Once coupled to Hib oligosaccharides the carrier proteins were injected in different mouse strains to verify the potential of their carrier effect. In BALB/c mice, an equivalent anti-Hib response was found when CRM197 and N10 were used as carrier proteins, whilst a lower response was found when N6 was used as carrier protein. This result was evident when the results were expressed using titres, while responder percentages failed to evidence the lower anti-Hib response obtained with the N6 protein carrier.

In C57BL/6 mice, the N6 protein gave a negative result, while positive results were obtained with CRM197 and N10, even if to a lower extent. These results were evident both using titres or responder percentages to express the results. When the results were expressed as a responder percentage, the high carrier effect of CRM197 and N10 was well evidenced with respect to N6, whose results were lower than 50% at day -34 and day -45 bleedings, after a comparable primary response (pre-2 bleeding, day 20).

Table II reports the results of the experiments in BALB/c and C57BL/6 mice.

In Swiss CDI mice, the titres obtained with the N10 carrier protein were equivalent to those obtained with CRM197. The anti-Hib titres increased after immunisation up to the 70th day, when a polysaccharide boost was given to assay whether or not an immunological memory was induced in the treated mice. No boost effect was observed with any carrier, although when CRM197 or N10 were used as carrier protein the titre did not decrease. In this mouse strain the immunisation with N6-Hib glycoconjugate give results very similar to the controls (polysaccharide and alum). The boost effect was not evidenced even in BALB/c mice that evoke a lower response with respect to Swiss CD1 mice.

The results are summarised in Table III.

Immunisation of different mice strains with Hib oligosaccharides conjugated to the artificial carrier proteins resulted in a good carrier effect exerted by N10 ,whilst N6 gave unsatisfactory results. This suggests that the size of the protein or the number of T cell epitopes has a high influence in providing T cell help to the oligosaccharides.

We used outbred CD1 mice to perform an immunogenicity experiment in which the carrier effect of N19 protein was compared to the carrier effects of N10 and CRM197. In addition, in order to explore potential carrier-induced immunosuppression phenomena, the three doses of N10-Hib, N19-Hib and CRM-Hib were given to groups of mice that did not received carrier priming and to groups of mice that one month before were primed with 50 μg of the respective unconjugated carrier (see materials and methods).

TABLE II

| BALB/c MICE | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | RESPONDER (%) | | | $A_{405}$ x 1000 (GMT's) | | |
| DAY | BLEEDING | N10-Hib | N5+146-Hib | CRM-Hib | N10-Hib | N5+146-Hib | CRM- Hib |
| 0 | PRE-IMMUNE | 0 | 0 | 0 | 10 | 17 | 12 |
| 20 | PRE-2 | 33.3 | 33.3 | 50 | 135 | 162 | 257 |
| 34 | POST-2/PRE-3 | 100 | 100 | 100 | 2022 | 1356 | 1969 |
| 45 | POST-3 | 100 | 100 | 100 | 1717 | 1368 | 1616 |
| C57BL/6 MICE | | | | | | | |
| | | RESPONDER (%) | | | $A_{405}$ x 1000 (GMT's) | | |
| DAY | BLEEDING | N10-Hib | N5+146-Hib | CRM-Hib | N10-Hib | N5+146-Hib | CRM- Hib |
| 0 | PRE-IMMUNE | 0 | 0 | 0 | 28 | 38 | 31 |
| 20 | PRE-2 | 83.3 | 83.3 | 83.3 | 136 | 192 | 609 |
| 34 | POST-2/PRE-3 | 83.3 | 33.3 | 100 | 1451 | 306 | 2612 |
| 45 | POST-3 | 100 | 33.3 | 100 | 1731 | 222 | 2240 |

TABLEIII

| SWISS CD1 MICE | | | | | | |
|---|---|---|---|---|---|---|
| | | TITRE GMT's ($A_{405nm} \times 10^3$) | | | | |
| DAY | BLEEDING | CRM-Hib | N5+146-Hib | N10-Hib | PsHib | ALUM |
| -1 | PRE-IMMUNISATION | 59 | 98 | 156 | 166 | 175 |
| 35 | POST-IMMUNISATION | 1577 | 471 | 1007 | 227 | 243 |
| 68 | PRE-BOOST | 2082 | 889 | 1789 | 590 | 461 |
| 85 | POST-BOOST | 2073 | 630 | 1767 | 364 | 479 |
| SWISS CD1 MICE | | | | | | |
| | | RESPONDER (%) | | | | |
| DAY | BLEEDING | CRM-Hib | N5+146-Hib | N10-Hib | PsHib | ALUM |
| -1 | PRE-IMMUNISATION | 0 | 0 | 0 | 0 | 0 |
| 35 | POST-IMMUNISATION | 100 | 50 | 62.5 | 0 | 0 |
| 68 | PRE-BOOST | 87.5 | 87.5 | 100 | 25 | 25 |
| 85 | POST-BOOST | 87.5 | 62.5 | 85.7 | 12.5 | 37.5 |

The schedule of the experiment was the following:

| | Days | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | -32 | **-30** | -2 | **0** | 14 | **15** | 27 | **28** | 45 |
| 1 | bleeding | **DT*** | bleeding | CRM-Hib | bleeding | CRM-Hib | bleeding | CRM-Hib | bleeding |
| 2 | bleeding | | bleeding | CRM-Hib | bleeding | CRM-Hib | bleeding | CRM-Hib | bleeding |
| 3 | bleeding | **N10** | bleeding | N10-Hib | bleeding | N10-Hib | bleeding | N10-Hib | bleeding |
| 4 | bleeding | | bleeding | N10-Hib | bleeding | N10-Hib | bleeding | N10-Hib | bleeding |
| 5 | bleeding | **N19** | bleeding | N19-Hib | bleeding | N19-Hib | bleeding | N19-Hib | bleeding |
| 6 | bleeding | | bleeding | N19-Hib | bleeding | N19-Hib | bleeding | N19-Hib | bleeding |

*For priming we used a chemically detoxified diphtheria toxin (DT: diphtheria toxoid) instead of the non toxic mutant (CRM-197) of diphtheria toxin.

The results are depicted in Figure 13. In unprimed mice the best anti-Hib titres were obtained using N19-Hib, whilst CRM-Hib and N10-Hib gave lower titres. According to the known direct proportion between the size of the carrier molecules and the exerted carrier effect, N19-Hib elicited a clearly improved anti-Hib response as compared to N10-Hib. In addition N19-Hib seems slightly superior also when compared to CRM-Hib suggesting the feasibility to substitute “classical” carrier proteins with the recombinant CD4+ polyepitope proteins. In contrast to the previous immunogenicity test performed on CD1 mice, were the carrier effects of N10 and CRM-197 were similar, in this new test the mean anti-Hib titre elicited by N10-Hib was notably lower than the one obtained with CRM-Hib.

In primed mice the best results were obtained with N19-Hib, which elicited a better response also when compared to the response obtained in unprimed mice, suggesting a potentiation due to the priming with N19 protein. A slight potentiation was also obtained after priming with N10. Conversely, anti-Hib response obtained with CRM-Hib in primed mice was notably lower of the response obtained in unprimed mice,

confirming the carrier induced immunosuppression often observed with the carriers in current use.

Since N10 and N19 contains five and ten tetanus toxoid T cell epitopes respectively, we subjected N10-Hib and N19-Hib to an immunogenicity test in CD1 mice primed with tetanus toxoid. The goal of this experiment was to check whether in primed mice the anti-Hib titers were improved in comparison to non-primed mice. Surprisingly, tetanus toxoid priming potentiated the immunoresponse to Hib in mice immunised with N10-Hib but not in mice that received N19-Hib (data not shown).

From the performed immunogenicity tests we can make the following few conclusions:

1. The carrier effect of the polyepitope protein is directly related to its size.
2. Recombinant polyepitope proteins N10 and N19 can parallel or exceed CRM-197 as carriers.
3. The polyepitope carrier proteins do not suffer of carrier induced suppression.

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## CLAIMS

1. A carrier protein comprising at least five CD4+ T cell epitopes.

2. A carrier protein according to claim 1, wherein the CD4+ epitopes are derived from a pathogenic bacterium or virus.

3. A carrier protein according to claim 1 or 2, wherein the CD4+ epitopes are derived from tetanus toxin, *Plasmodium falciparum* circumsporozite protein, hepatitis B surface antigen, hepatitis B nuclear core protein, influenza matrix protein, influenza haemagglutinin, diptheria toxoid, diptheria toxin mutant CRM 197, group B *Neisseria meningitidis* outer membrane protein complex, pertussis toxin or heat shock protein 70.

4. A carrier protein according to any one of the preceding claims wherein the CD4+ epitopes are selected from the P23TT, P32TT, P21TT, PfCs, P30TT, P2TT, HBVnc, HA, HbsAg, MT and hsp70 CD4+ epitopes.

5. A carrier protein according to claim 1, that comprises the P23TT, P32TT, P21TT, PfCs, P30TT, P2TT, HBVnc, HA, HbsAg and MT CD4+ epitopes.

6. A carrier protein according to claim 1, that comprises the P23TT, P32TT, P21TT, PfCs, P30TT, P2TT, HBVnc, HA, HbsAg, MT and hsp70 CD4+ epitopes.

7. A carrier protein according to claim 1, that comprises the P23TT, P32TT, P21TT, PfCs, P30TT and P2TT CD4+ epitopes.

8. A carrier protein according any one of the preceding claims, wherein the CD4+ epitopes are human CD4+ epitopes.

9. A carrier protein which comprises one or more of N6, N10 or N19 proteins.

10. A carrier protein according to any one of the preceding claims in an oligomeric form.

11. A carrier protein according to any one of the preceding claims, conjugated to polysaccharide.

12. A carrier protein according to claim 11, wherein the polysaccharide is an *Haemophilus influenzae* type B polysaccharide.

13. A carrier protein according to claim 11, wherein the polysaccharide is derived from any one of the following organisms: *S. pneumoniae, N. meningitidis, S. aureus, Klebsiella*, or *S. typhimurium*.

14. A carrier protein according to any one of claims 11-13 where the polysaccharide is conjugated to protein by a covalent linkage.

15. A carrier protein according to claim any one of claims 11-13, wherein the polysaccharide is conjugated to protein by reductive amination.

16. A carrier protein according to any one of claims 11-15, wherein there are between two and ten protein units for each polysaccharide unit.

17. A carrier protein according to any one of claims 1 to 16 for use as a pharmaceutical.

18. Use of the carrier protein according to any one of claims 1 to 16 as a pharmaceutical.

19. The carrier protein according to any one of claims 1 to 16 for use as a vaccine or as a component of a vaccine.

20. Use of a carrier protein according to any one of claims 1 to 16 as a vaccine or vaccine component.

21. A vaccine comprising a carrier protein according to any one of claims 1 to 16.

22. A method of production of vaccination comprising introducing into a mammal, preferably a human, a carrier protein according to any one of claims 1 to 16.

23. The carrier protein according to any one of claims 1 to 16 for use as a protective immunogen in the control of diseases caused by encapsulated bacteria.

24. A nucleic acid molecule which encodes a carrier protein according to any one of claims 1 to 10.

25. The nucleic acid molecule of claim 24 which comprises DNA.

26. A cloning or expression vector comprising a nucleic acid molecule according to either of claims 24-25.

27. A host cell transformed or transfected with the vector of claim 26.

28. A transgenic animal that has been transformed by a nucleic acid molecule according to either of claims 24 or 25 or by a vector according to claim 26.

29. A method of preparing a carrier protein according to any one of claims 1 to 10, comprising expressing a vector according to claim 26 in a host cell and culturing said host cell under conditions where said protein is expressed, and recovering said protein thus expressed.

30. A method of production of a carrier protein according to any one of claims 1-10 comprising the steps of:

a) constructing oligonucleotide molecules that encode peptide epitopes;

b) annealing the oligonucleotide molecules to form duplexes;

c) introducing the oligonucleotide duplexes into an expression vector so as to encode a fusion protein;

d) introducing the expression vector into a host cell to allow expression of the fusion protein; and

e) isolating the fusion protein produced from a culture of said host cells.

31. The method of claim 30, further comprising the step of conjugating the fusion protein to polysaccharide.

32. The method of claim 29, wherein the host cell is an *E. coli* bacterium.

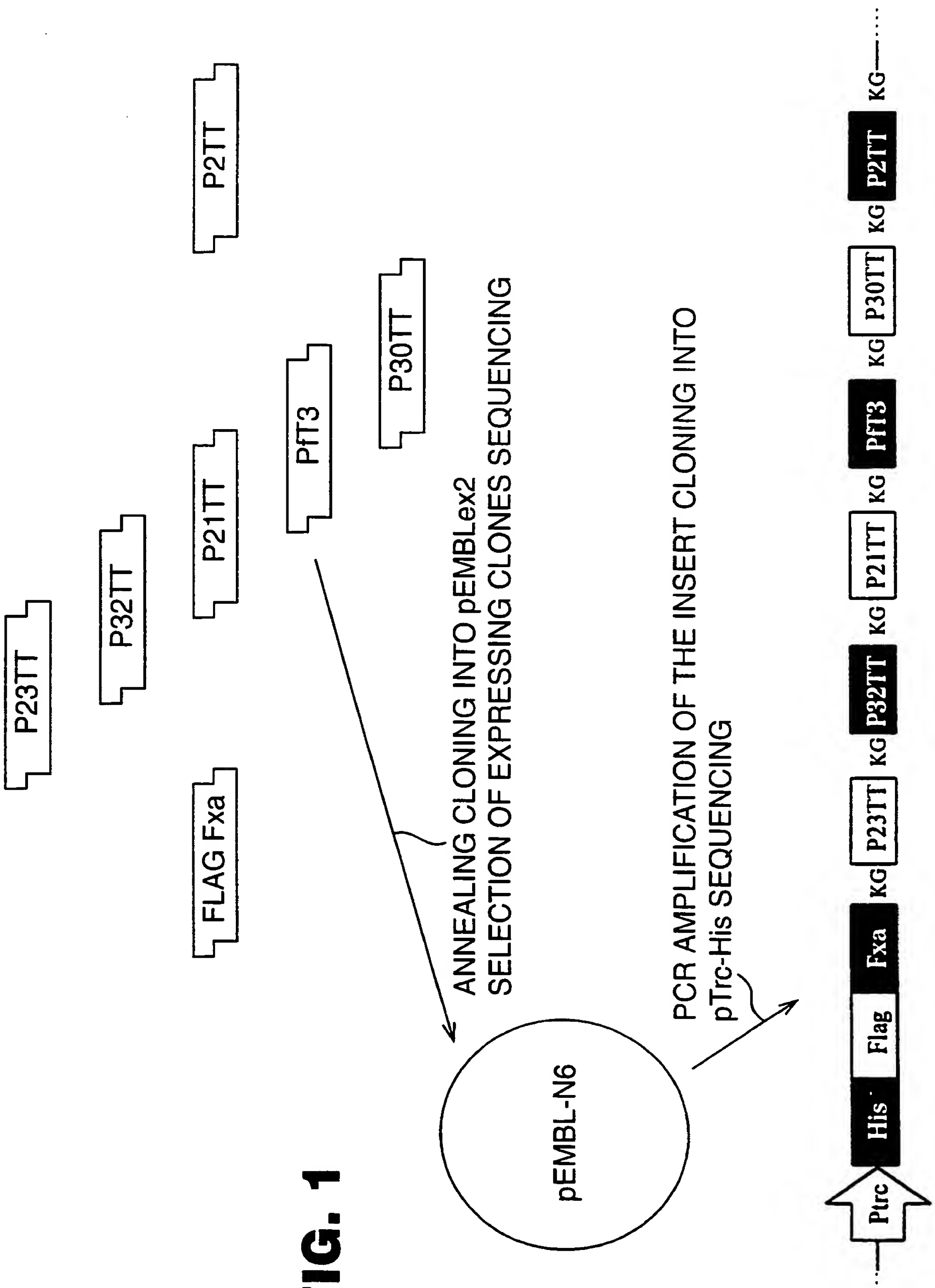

P23TT
P32TT
P21TT
PfT3
P30TT
P2TT
FLAG Fxa
ANNEALING CLONING INTO pEMBLex2
SELECTION OF EXPRESSING CLONES SEQUENCING
pEMBL-N6
PCR AMPLIFICATION OF THE INSERT CLONING INTO
pTrc-His SEQUENCING
Ptrc
His
Flag
Fxa
KG
P23TT
KG
P32TT
KG
P21TT
KG
PfT3
KG
P30TT
KG
P2TT
KG

FIG. 1

# FIG. 2


ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG GAT TAC AAG GAC GAC GAT GAT ATC GAA GGT CGC
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Asp Tyr Lys Asp Asp Asp Asp Ile Glu Gly Arg

AAA GGT GTT TCC ATC GAC AAA TTC CGT ATC TTC TGC AAA GCT AAC CCG AAA AAA GGT CTG AAA TTC ATC ATC AAA CGT
Lys Gly Val Ser Ile Asp Lys Phe Arg Ile Phe Cys Lys Ala Asn Pro Lys Lys Gly Leu Lys Phe Ile Ile Lys Arg

TAC ACC CCG AAC AAC GAA ATC GAC TCC AAA GGT ATC CGT GAA GAC AAC AAC ATC ACC CTG AAA CTG GAC CGT TGC AAC
Tyr Thr Pro Asn Asn Glu Ile Asp Ser Lys Gly Ile Arg Glu Asp Asn Asn Ile Thr Leu Lys Leu Asp Arg Cys Asn

AAC AAA GGT GAA AAG AAG ATC GCT AAA ATG GAA AAA GCT TCT TCT GTT TTC AAC GTT GTT AAC TCT AAA GGT TTC AAC
Asn Lys Gly Glu Lys Lys Ile Ala Lys Met Glu Lys Ala Ser Ser Val Phe Asn Val Val Asn Ser Lys Gly Phe Asn

AAC TTC ACC GTT TCC TTC TGG CTG CGT GTT CCG AAA GTT TCC GCT TCC CAC CTG GAA AAA GGT CAG TAC ATC AAA GCT
Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Lys Gly Gln Tyr Ile Lys Ala

AAC TCC AAA TTC ATC GGT ATC ACC GAA AAA GGT GGA TCC TAA
Gln Ser Lys Phe Ile Gly Ile Thr Glu Lys Gly Gly Ser End

# FIG. 2(CONTD.)

## N10


ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG GAT TAC AAG GAC GAC GAT GAT ATC GAA GGT CGC
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Asp Tyr Lys Asp Asp Asp Asp Ile Glu Gly Arg

AAA GGT GTT TCC ATC GAC AAA TTC CGT ATC TTC TGC AAA GCT AAC CCG AAA AAA GGT CTG AAA TTC ATC ATC AAA CGT
Lys Gly Val Ser Ile Asp Lys Phe Arg Ile Phe Cys Lys Ala Asn Pro Lys Lys Gly Leu Lys Phe Ile Ile Lys Arg

TAC ACC CCG AAC AAC GAA ATC GAC TCC AAA GGT ATC CGT GAA GAC AAC AAC ATC ACC CTG AAA CTG GAC CGT TGC AAC
Tyr Thr Pro Asn Asn Glu Ile Asp Ser Lys Gly Ile Arg Glu Asp Asn Asn Ile Thr Leu Lys Leu Asp Arg Cys Asn

AAC AAA GGT GAA AAG AAG ATC GCT AAA ATG GAA AAA GCT TCT TCT GTT TTC AAC GTT GTT AAC TCT AAA GGT TTC AAC
Asn Lys Gly Glu Lys Lys Ile Ala Lys Met Glu Lys Ala Ser Ser Val Phe Asn Val Val Asn Ser Lys Gly Phe Asn

AAC TTC ACC GTT TCC TTC TGG CTG CGT GTT CCG AAA GTT TCC GCT TCC CAC CTG GAA AAA GGT CAG TAC ATC AAA GCT
Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Lys Gly Gln Tyr Ile Lys Ala

AAC-TCC AAA TTC ATC GGT ATC ACC GAA AAA GGT GGA TCT CCG CAT CAT ACC GCG CTG CGC CAG GCG ATT CTG TGC TGG
Gln Ser Lys Phe Ile Gly Ile Thr Glu **Lys Gly** Gly Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp

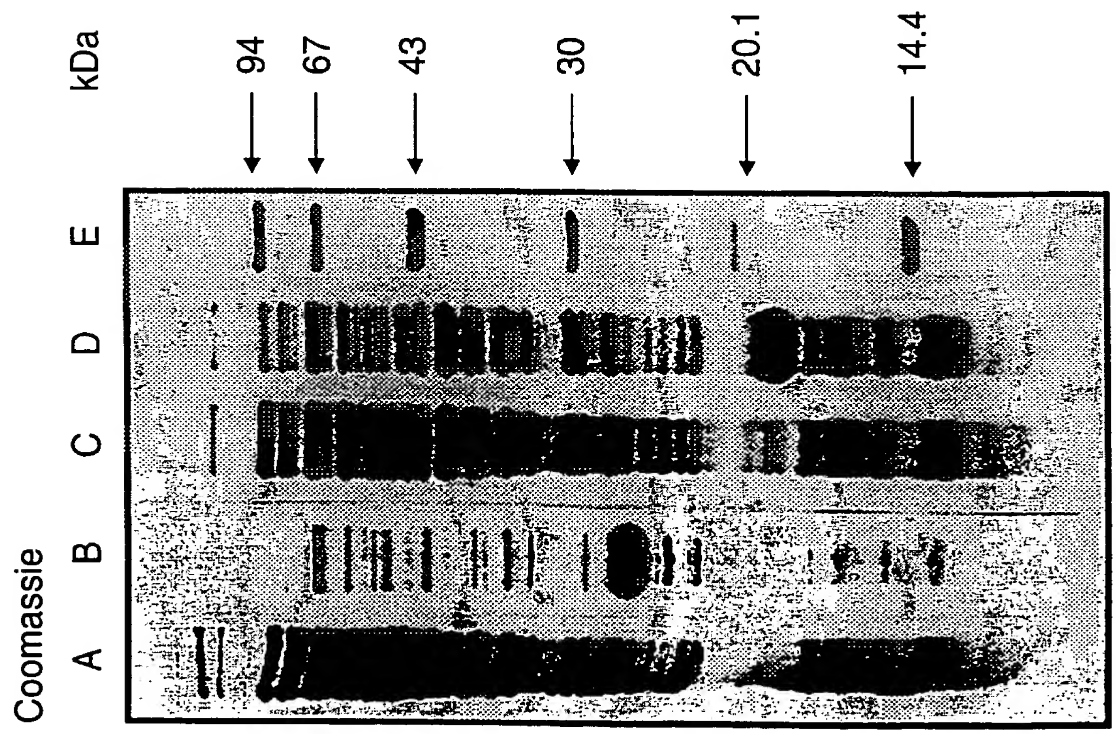
GGC GAA CTG ATG ACC CTG GCG AAA GGA TCT CCG AAA TAT GTG AAA CAG AAC ACC CTG AAA CTG GCG ACC AAA GGA TCG
Gly Glu Leu Met Thr Leu Ala **Lys Gly** Ser Pro Lys Tyr Val Lys Gln Asn Thr Leu Lys Leu Ala Thr **Lys Gly** Ser

TTT TTT CTG CTG ACC CGC ATT CTG ACC ATT CCG CAG TCT CTG GAT AAA GGC TAT TCT GGC CCG CTG AAA GCG GAA ATT
Phe Phe Leu Leu Thr Arg Ile Leu Thr Ile Pro Gln Ser Leu Asp **Lys Gly** Tyr Ser Gly Pro Leu Lys Ala Glu Ile

GCG CAG CGC CTG GAA GAT GTG AAA GGA TCC TAA
Ala Gln Arg Leu Glu Asp Val **Lys Gly** Ser End

**FIG. 2**(CONTD.)

# FIG. 3


Coomassie
A B C D E
kDa
94
67
43
30
20.1
14.4

# FIG. 4

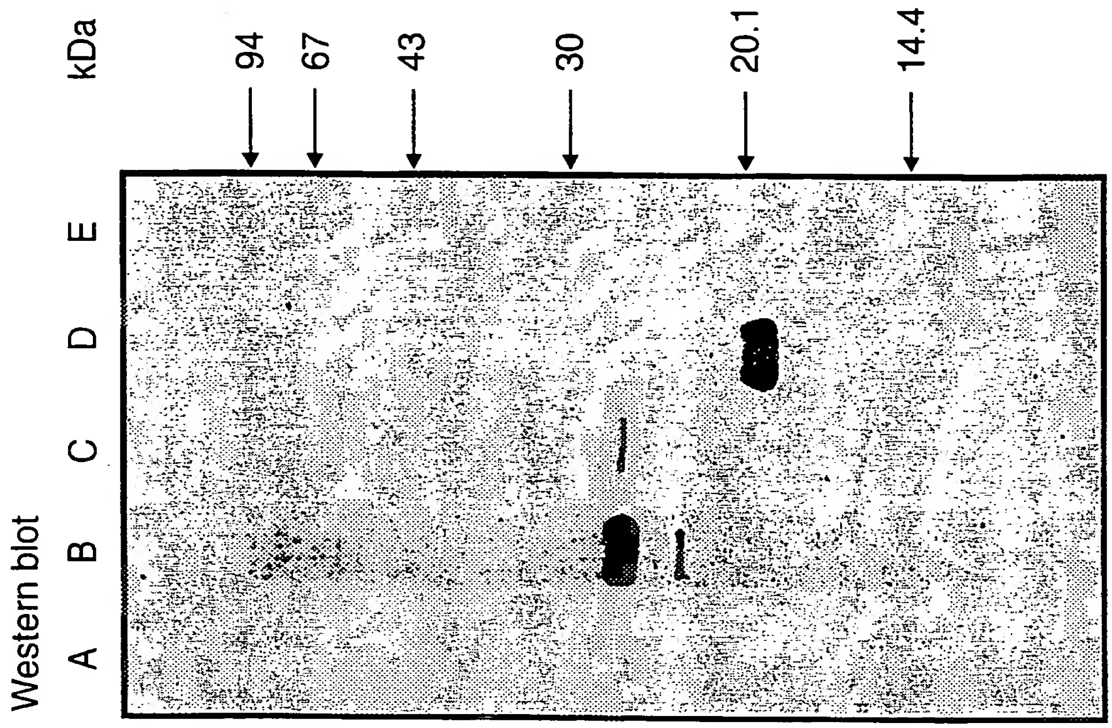

Western blot
A B C D E
kDa
94
67
43
30
20.1
14.4

**FIG. 6**

Western blot

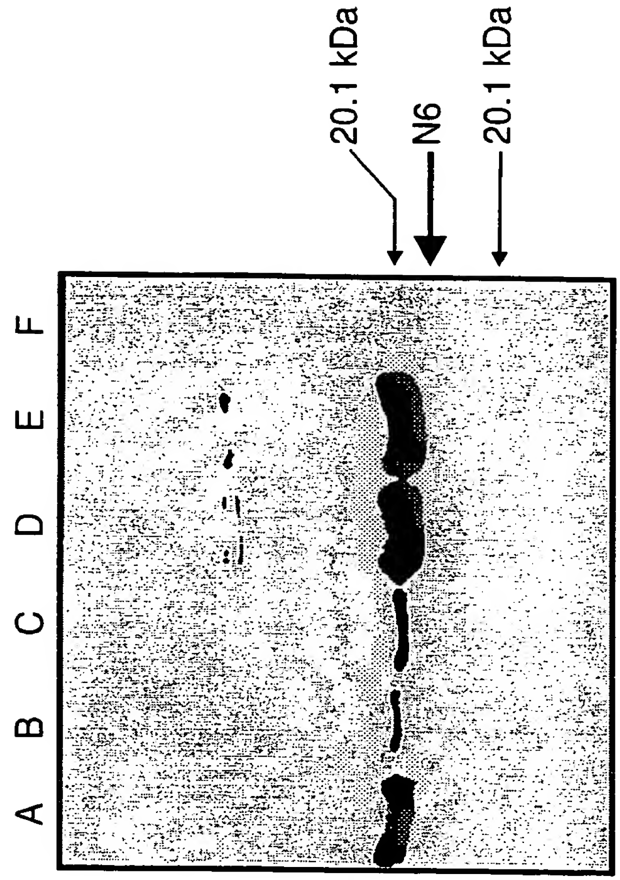
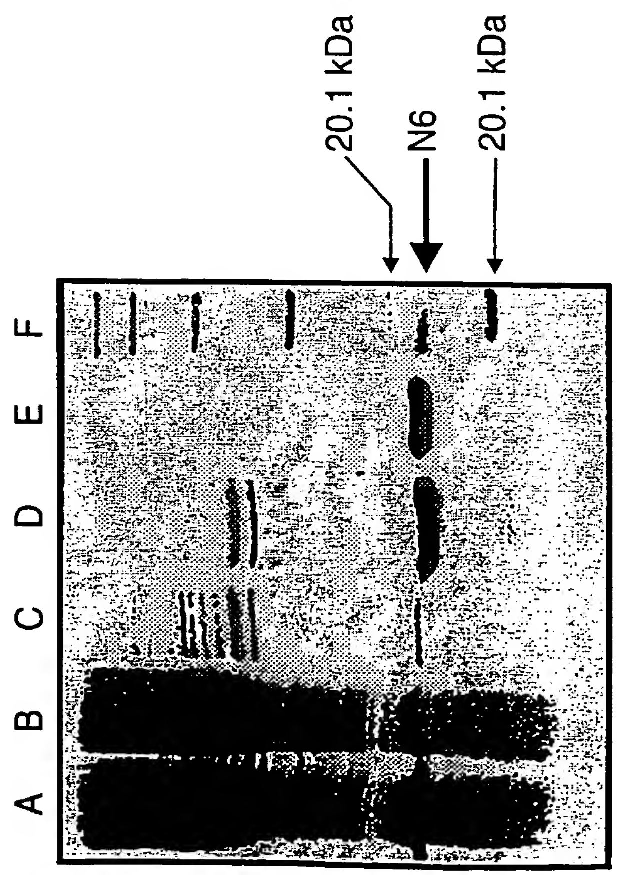


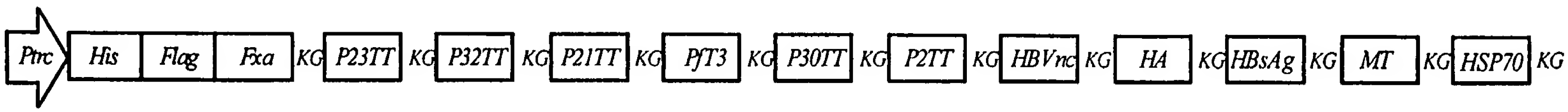

**FIG. 5**

Coomassie



# FIG. 7

N11




ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG GAT TAC AAG GAC GAC GAT GAT ATC GAA GGT CGC
MET GLY GLY SER HIS HIS HIS HIS HIS HIS GLY MET ALA SER MET ASP TYR LYS ASP ASP ASP ASP ILE GLU GLY ARG

AAA GGT GTT TCC ATC GAC AAA TTC CGT ATC TTC TGC AAA GCT AAC CCG AAA AAA GGT CTG AAA TTC ATC ATC AAA CGT
**LYS GLY** VAL SER ILE ASP LYS PHE ARG ILE PHE CYS LYS ALA ASN PRO LYS **LYS GLY** LEU LYS PHE ILE ILE LYS ARG

TAC ACC CCG AAC AAC GAA ATC GAC TCC AAA GGT ATC CGT GAA GAC AAC AAC ATC ACC CTG AAA CTG GAC CGT TGC AAC
TYR THR PRO ASN ASN GLU ILE ASP SER LYS GLY ILE ARG GLU ASP ASN ASN ILE THR LEU LYS LEU ASP ARG CYS ASN

AAC AAA GGT GAA AAG AAG ATC GCT AAA ATG GAA AAA GCT TCT TCT GTT TTC AAC GTT GTT AAC TCT AAA GGT TTC AAC
ASN **LYS GLY** GLU LYS LYS ILE ALA LYS MET GLU LYS ALA SER SER VAL PHE ASN VAL VAL ASN SER **LYS GLY** PHE ASN

AAC TTC ACC GTT TCC TTC TGG CTG CGT GTT CCG AAA GTT TCC GCT TCC CAC CTG GAA AAA GGT CAG TAC ATC AAA GCT
ASN PHE THR VAL SER PHE TRP LEU ARG VAL PRO LYS VAL SER ALA SER HIS LEU GLU **LYS GLY** GLN TYR ILE LYS ALA

AAC TCC AAA TTC ATC GGT ATC ACC GAA AAA GGT GGA TCT CCG CAT CAT ACC GCG CTG CGC CAG GCG ATT CTG TGC TGG
GLN SER LYS PHE ILE GLY ILE THR GLU **LYS GLY** GLY SER PRO HIS HIS THR ALA LEU ARG GLN ALA ILE LEU CYS TRP

GGC GAA CTG ATG ACC CTG GCG AAA GGA TCT CCG AAA TAT GTG AAA CAG AAC ACC CTG AAA CTG GCG ACC AAA GGA TCG
GLY GLU LEU MET THR LEU ALA **LYS GLY** SER PRO LYS TYR VAL LYS GLN ASN THR LEU LYS LEU ALA THR **LYS GLY** SER

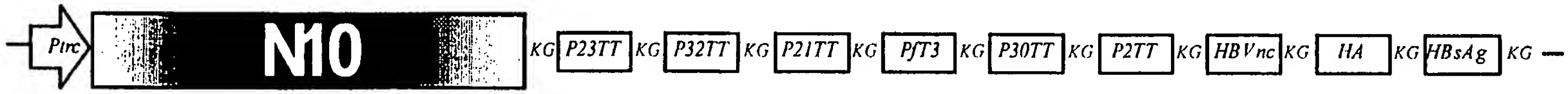
TTT TTT CTG CTG ACC CGC ATT CTG ACC ATT CCG CAG TCT CTG GAT AAA GGC TAT TCT GGC CCG CTG AAA GCG GAA ATT
PHE PHE LEU LEU THR ARG ILE LEU THR ILE PRO GLN SER LEU ASP **LYS GLY** TYR SER GLY PRO LEU LYS ALA GLU ILE

GCG CAG CGC CTG GAA GAT GTG AAA GGA TCT CAG CCG TCT GTT CAG ATT CAG GTG TAT CAG GGT GAA CGT GAA ATC GCA
ALA GLN ARG LEU GLU ASP VAL **LYS GLY** SER GLN PRO SER VAL GLN ILE GLN VAL TYR GLN GLY GLU ARG GLU ILE ALA

TCT CAT AAC AAA GGA TCC TAA
SER HIS ASN LYS GLY SER END

**FIG. 7**(CONTD.)

# FIG. 8




ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG GAT TAC AAG GAC GAC GAT GAT ATC GAA GGT CGC
MET GLY GLY SER HIS HIS HIS HIS HIS HIS GLY MET ALA SER MET ASP TYR LYS ASP ASP ASP ASP ILE GLU GLY ARG

AAA GGT GTT TCC ATC GAC AAA TTC CGT ATC TTC TGC AAA GCT AAC CCG AAA AAA GGT CTG AAA TTC ATC ATC AAA CGT
**LYS GLY** VAL SER ILE ASP LYS PHE ARG ILE PHE CYS LYS ALA ASN PRO LYS **LYS GLY** LEU LYS PHE ILE ILE LYS ARG

TAC ACC CCG AAC AAC GAA ATC GAC TCC AAA GGT ATC CGT GAA GAC AAC AAC ATC ACC CTG AAA CTG GAC CGT TGC AAC
TYR THR PRO ASN ASN GLU ILE ASP SER **LYS GLY** ILE ARG GLU ASP ASN ASN ILE THR LEU LYS LEU ASP ARG CYS ASN

AAC AAA GGT GAA AAG AAG ATC GCT AAA ATG GAA AAA GCT TCT TCT GTT TTC AAC GTT GTT AAC TCT AAA GGT TTC AAC
ASN **LYS GLY** GLU LYS LYS ILE ALA LYS MET GLU LYS ALA SER SER VAL PHE ASN VAL VAL ASN SER **LYS GLY** PHE ASN

AAC TTC ACC GTT TCC TTC TGG CTG CGT GTT CCG AAA GTT TCC GCT TCC CAC CTG GAA AAA GGT CAG TAC ATC AAA GCT
ASN PHE THR VAL SER PHE TRP LEU ARG VAL PRO LYS VAL SER ALA SER HIS LEU GLU **LYS GLY** GLN TYR ILE LYS ALA

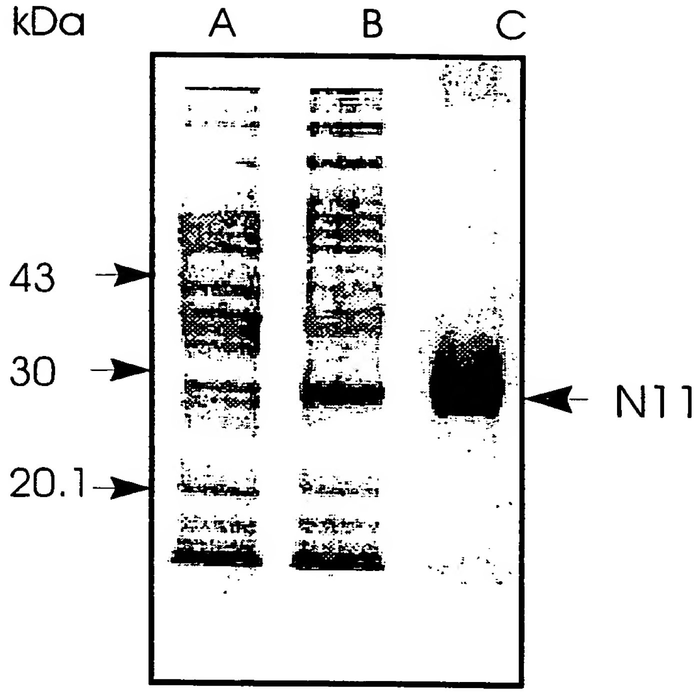
AAC TCC AAA TTC ATC GGT ATC ACC GAA AAA GGT GGA TCT CCG CAT CAT ACC GCG CTG CGC CAG GCG ATT CTG TGC TGG
GLN SER LYS PHE ILE GLY ILE THR GLU **LYS GLY** GLY SER PRO HIS HIS THR ALA LEU ARG GLN ALA ILE LEU CYS TR

GGC GAA CTG ATG ACC CTG GCG AAA GGA TCT CCG AAA TAT GTG AAA CAG AAC ACC CTG AAA CTG GCG ACC AAA GGA TCG
GLY GLU LEU MET THR LEU ALA **LYS GLY** SER PRO LYS TYR VAL LYS GLN ASN THR LEU LYS LEU ALA THR **LYS GLY** SER

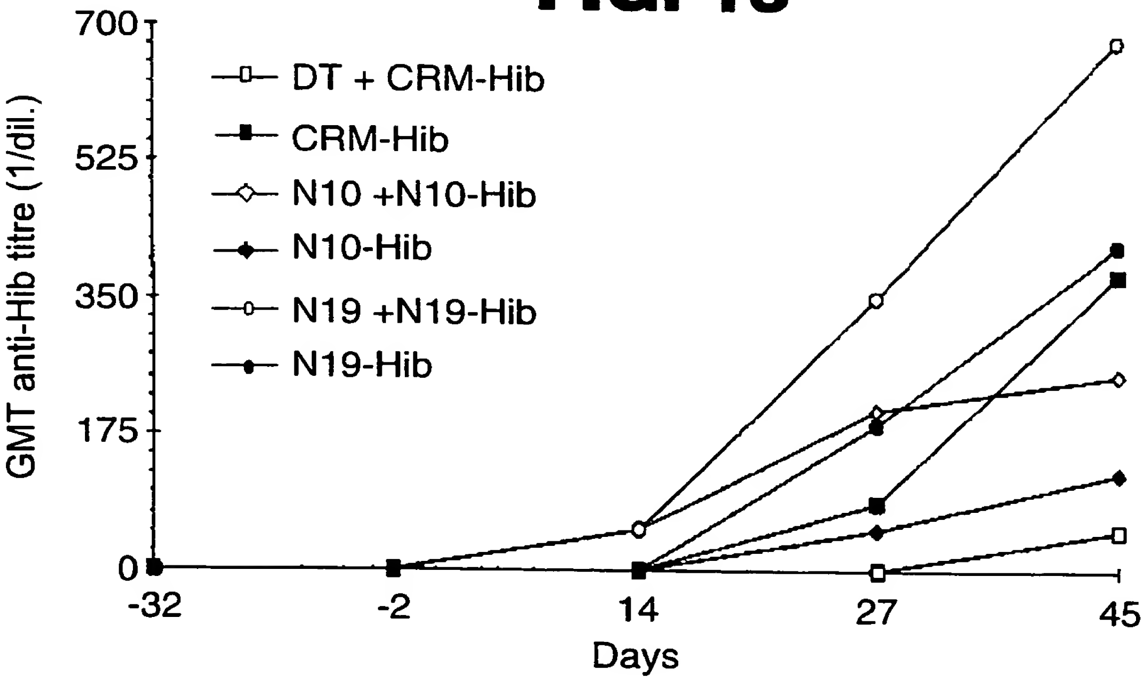
```
TTT TTT CTG CTG ACC CGC ATT CTG ACC ATT CCG CAG TCT CTG GAT AAA GGC TAT TCT GGC CCG CTG AAA GCG GAA ATT
PHE PHE LEU LEU THR ARG ILE LEU THR ILE PRO GLN SER LEU ASP LYS GLY TYR SER GLY PRO LEU LYS ALA GLU ILE

GCG CAG CGC CTG GAA GAT GTG AAA GGA TCT GTT TCC ATC GAC AAA TTC CGT ATC TTC TGC AAA GCT AAC CCG AAA AAA
ALA GLN ARG LEU GLU ASP VAL LYS GLY SER VAL SER ILE ASP LYS PHE ARG ILE PHE CYS LYS ALA ASN PRO LYS LYS

GGT CTG AAA TTC ATC ATC AAA CGT TAC ACC CCG AAC AAC GAA ATC GAC TCC AAA GGT ATC CGT GAA GAC AAC AAC ATC
GLY LEU LYS PHE ILE ILE LYS ARG TYR THR PRO ASN ASN GLU ILE ASP SER LYS GLY ILE ARG GLU ASP ASN ASN ILE

ACC CTG AAA CTG GAC CGT TGC AAC AAC AAA GGT GAA AAG AAG ATC GCT AAA ATG GAA AAA GCT TCT TCT GTT TTC AAC
THR LEU LYS LEU ASP ARG CYS ASN ASN LYS GLY GLU LYS LYS ILE ALA LYS MET GLU LYS ALA SER SER VAL PHE ASN

GTT GTT AAC TCT AAA GGT TTC AAC AAC TTC ACC GTT TCC TTC TGG CTG CGT GTT CCG AAA GTT TCC GCT TCC CAC CTG
VAL VAL ASN SER LYS GLY PHE ASN ASN PHE THR VAL SER PHE TRP LEU ARG VAL PRO LYS VAL SER ALA SER HIS LEU

GAA AAA GGT CAG TAC ATC AAA GCT AAC TCC AAA TTC ATC GGT ATC ACC GAA AAA GGT GGA TCT CCG CAT CAT ACC GCG
GLU LYS GLY GLN TYR ILE LYS ALA GLN SER LYS PHE ILE GLY ILE THR GLU LYS GLY GLY SER PRO HIS HIS THR ALA

CTG CGC CAG GCG ATT CTG TGC TGG GGC GAA CTG ATG ACC CTG GCG AAA GGA TCT CCG AAA TAT GTG AAA CAG AAC ACC
LEU ARG GLN ALA ILE LEU CYS TRP GLY GLU LEU MET THR LEU ALA LYS GLY SER PRO LYS TYR VAL LYS GLN ASN THR

CTG AAA CTG GCG ACC AAA GGA TCG TTT TTT CTG CTG ACC CGC ATT CTG ACC ATT CCG CAG TCT CTG GAT AAA GGA TCC
LEU LYS LEU ALA THR LYS GLY SER PHE PHE LEU LEU THR ARG ILE LEU THR ILE PRO GLN SER LEU ASP LYS GLY SER

TAA
END
```

**FIG. 8**(CONTD.)

# FIG. 9


kDa
A
B
C
43
30
20.1
N11

# FIG. 13


700
525
350
175
0
GMT anti-Hib titre (1/dil.)
DT + CRM-Hib
CRM-Hib
N10 +N10-Hib
N10-Hib
N19 +N19-Hib
N19-Hib
-32
-2
14
27
45
Days

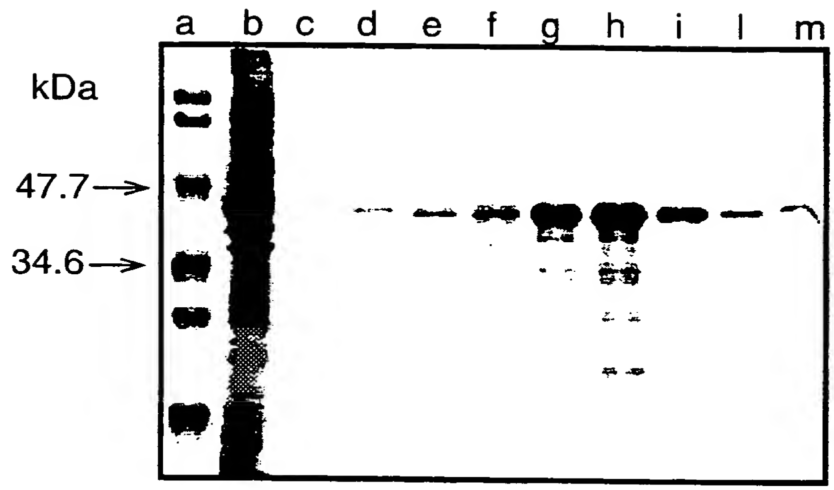



FIG. 10A

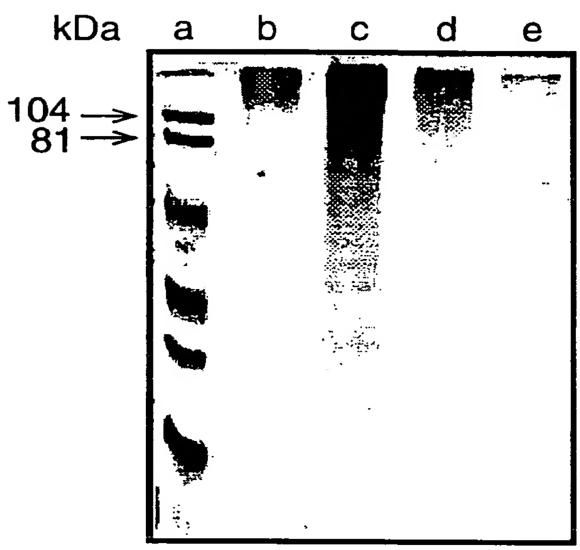



FIG. 10B

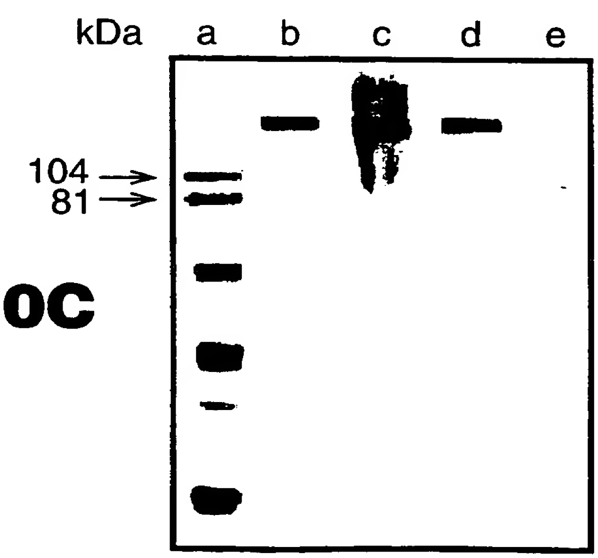



FIG. 10C

FIG. 11

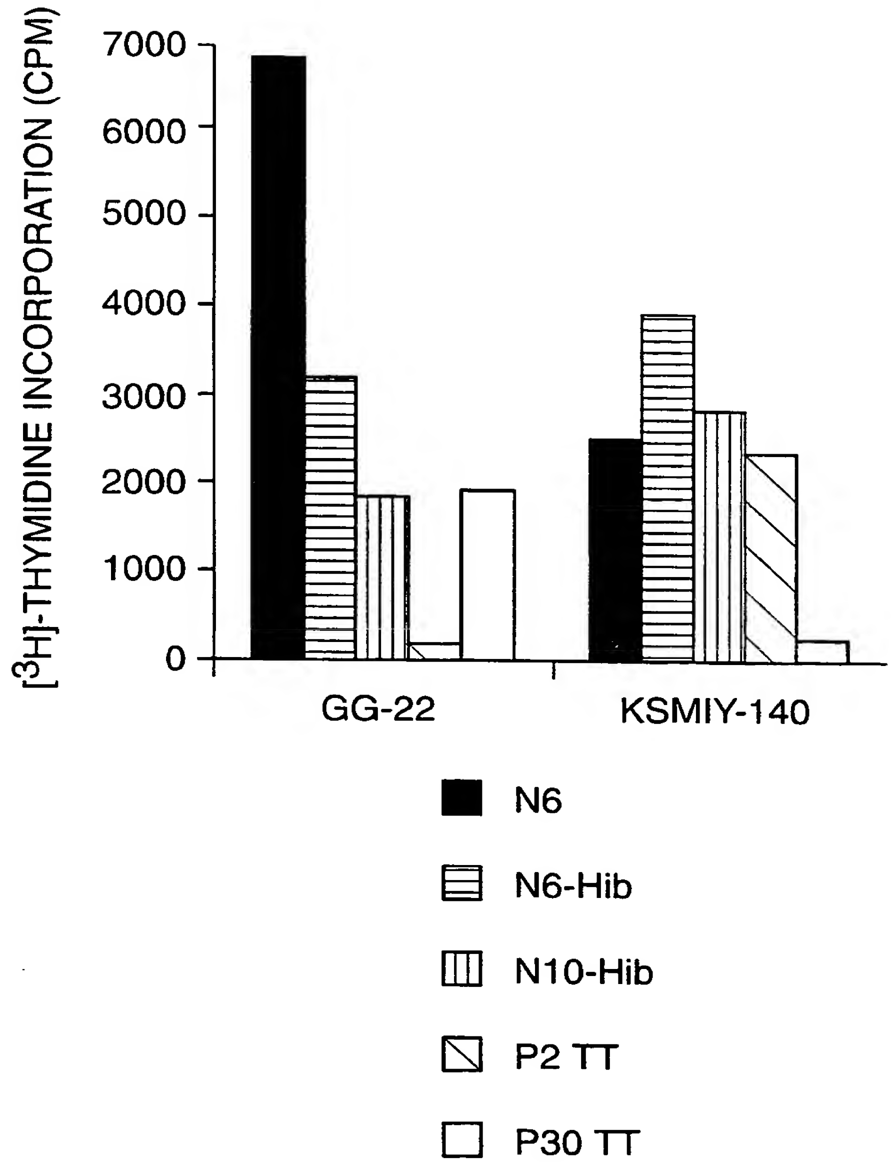
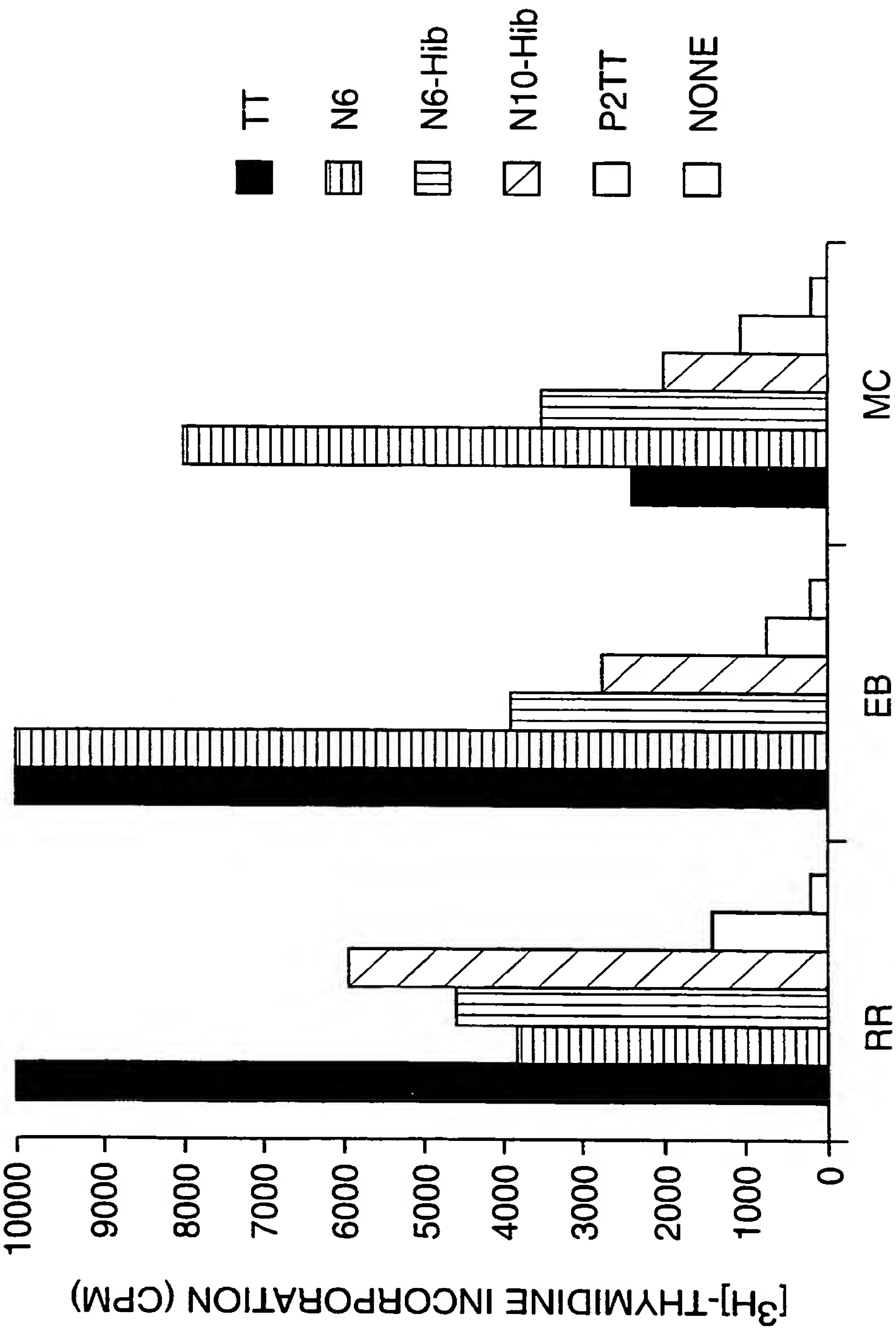

[3H]-THYMIDINE INCORPORATION (CPM)
7000
6000
5000
4000
3000
2000
1000
0
GG-22
KSMIY-140
N6
N6-Hib
N10-Hib
P2 TT
P30 TT

FIG. 12


TT
N6
N6-Hib
N10-Hib
P2TT
NONE
[3H]-THYMIDINE INCORPORATION (CPM)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
RR
EB
MC